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(71) Applicant: BRISTOL-MYERS SQUIBB COMPANY [US/US]; 345 Park Avenue, New York, NY 10154 (US).

(72) Inventors: THOMAS, Elaine, Kinney ; 2015 23rd Avenue West, Seattle, WA 98199 (US). CHEN, Lieping ; 300 10th Avenue #A409, Seattle, WA 98122 (US). BLAKE, James ; 201 Galer Avenue #2433, Seattle, WA 98109 (US). HELLSTRÖM, Karl, Erik ; ; HELLSTRÖM, Ingegerd ; 3925 N.E. Surber Drive, Seattle, WA 98105 (US). HU, Shiu-Lok ; 17018 N.E. 133rd Street, Redmond, WA 98052 (US).

(74) Agent: POOR, Brian, W.; Bristol-Myers Squibb Company, 3005 First Avenue, Seattle, WA 98121 (US).

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(57) Abstract

Peptides, antibodies and recombinanant expression systems or cells that contain and express a DNA insert of HPV encoding a region of a papilloma induced or a papibilloma protein, such as E6 or E7, are produced. Compositions containing these peptides, antibodies and/or recombinant cells are e utilized as immunogenic compositions and in methods for inhibiting and treating HPV infection and tumor initiation and progreression. Specific peptides and recombinant cells, such as vaccinia virus and tumor cells, that express epitopic regions of the HPVM16 E6 or E7 nucleoprotein are particularly described.

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# HUMMAN PAPILLOMA VIRAL PROTEIN EXPRESSION FOR USE IN VACCINE COMPOSITIONS

This aapplication is a continuation-in-part of co-pending U.S. patent applications SSerial Number 007,230, filed January 27, 1987, which is a continuation-iriin-part of application Serial Number 827,313, filed February 7, 1986, now abbandoned; and Serial Number 905,217 filed September 9, 1987, which is a ccontinuation-in-part of application Serial Number 842,984, filed March 27,19866, now abandoned.

## FIELD OF INVENTION

The ppresent invention is directed to recombinant cells, peptides, antibodies, commpositions and methods that can be utilized for the inhibition and treatment of huuman papilloma virus (HPV) infection and cell transformation.

Recombbinant cells that contain, and express, a DNA insert that encodes a region of aan HPV protein or a peptide induced by an HPV gene in a mammalian celell are produced, such as recombinant vaccinia virus that expresses an epitopic reggion of the E6 or E7 nucleoprotein gene product of HPV or a transfected or r recombinant virus-infected, reconstructed fibroblast, epithelial cell, lymphocyyte or tumor cell that contains and expresses a region of the E6 or E7 nucleoprotein gene product of HPV. Specific peptides have been prepared that correspondd to epitopic regions of HPV16 E6 and E7 proteins and these peptides have been utilized in immunogenic compositions and vaccines.

Therapeutic aand prophylactic methods are described for the inhibition and regression of I HPV infections and tumor development in patients.

## BACKGROUND OF THE INVENTION

Work : with experimental animals, particularly rodents, has shown that most tumors irinduced by oncogenic viruses express antigens encoded by the viral genome, and i that immunization with these antigens can lead to rejection of a subsequent chhallenge of tumor cells induced by the same virus. Although much of this work wwas done with laboratory strains of virus, such as SV40, polyoma virus, and Friciend, Moloney, or Rauscher murine leukemia viruses, horizontal and vertical trransmission of oncogenic viruses in nature has been demonstrated; indeed a commmercial vaccine against virus-induced feline leukemia and sarcoma is now availablble.

By corntrast, a viral etiology of most human cancer has not been demonstrated. . Possible exceptions are hepatitis virus (hepatoma), Epstein Barr virus (nasophharyngeal carcinoma), and human papilloma virus (HPV16) (cervical carcicinoma). However, during the past two decades it has been established thaat some human tumor cells express tumor antigens, i.e., antigens that distinguisssh the tumor cells from their normal cellular counterparts. Some patients mounnt cell-mediated or humoral immune responses against these antigens (Hellilström et al. (1968) Nature, 220:1352; Morton et al. (1968) Science 162:1279-1281; Shiku et al. (1976) J. Exp. Med. 144: 873-881). Some of the tatargets of these immune responses are oncofetal or differentiation antigens encodded by the human genome (Hellström et al. (1970) Int. J. Cancer 6:346-351).

Until rerecently the molecular nature of the tumor antigens was unknown, and the degree e of tumor specificity of the immunological reactions was unclear.

Attempts to utililize this information in developing cancer diagnostic assays or cancer therapiees have been largely unsuccessful. Because spontaneous tumor regressions aree extremely rare, one may also conclude that the immune responses demoonstrated in vitro were ineffective in vivo. For example, while antibodies and l lymphocytes obtained from a cancer patient may be effective in killing tumor ceells in vitro, the immune response of the same cancer patient is insufficient in vivivo to prevent tumor development.

The devvelopment by Kohler and Milstein of the monoclonal antibody technique (1975'5, Nature 256:495-497) lead to intensified searches for human tumor antigens, , because it provided the means to define such antigens, both at the molecular l level and with respect to specificity (Hellström and Brown, (1979) in The AAntigens, M. Sela, ed., Academic Press, Vol. V:1-66).

Over thee past several years large numbers of tumor-associated antigens have been descrribed, most of which have been defined by mouse monoclonal antibodies (Reisisfeld and Sell, eds., (1985) in Monoclonal Antibodies and Cancer Therapyy, UCLA Symposia on Molecular and Cellular Biology, New Series, Vol. 27,', Alan R. Liss, Inc. New York, pp. 1-609). Although virtually all of the antiggens which have been well characterized have proven to be oncofetal or diffferentiation antigens, and their specificity for tumors has been found to be quaantitative rather than qualitative, several antigens are sufficiently specific for neopplastic versus normal cells (generally corresponding to a factor of 10 to 1.000 titimes) to be used as potential targets for identifying tumor cells and for therapy.. Human monoclonal antibodies to tumor antigens have also been obtained (CCote et al. (1983) Proc. Natl. Acad. Sci. 80:2026-2030). This supports the preeviously cited evidence that some cancer patients mount an immune reactionn to their tumors. Many of these tumor antigens are encoded by

the human genome, while in some cases these tumor antigens are the result of endogenous or exogenous viral infection.

Human papilloma viruses are well-known infective agents that produce epithelial neoplasia, such as warts and papillomas, in their hosts. Common hand warts and plantar warts are the most frequent skin lesions in humans; however, squamous cell carcinomas and genital cancers in both males and females are also commonly associated with certain strains of HPV infection.

The papilloma virus genome contains a double stranded, circular supercoiled DNA molecule having a molecular weight of about 5,000 kilodaltons (kDa). This genome encodes between 8 and 10 proteins, this number being uncertain because a function or protein product has not yet been assigned to each of the open-reading frames (ORFs) of the genome. The ORFs produced early in replication were originally designated with an E, and those produced late with an L. This designation however, has not held up, and it has been found that some E gene products are produced early and late in infection.

Infection by HPV is strongly associated with cervical cancer and other anogenital carcinomas in humans. (Zur Hausen et al. (1989) Cancer Res. 49:4677-4681; Galloway et al. (1989) Adv. Virus Res. 37:125-171). One HPV type, HPV16,5, is commonly linked with severe dysplasias and with cervical cancers. (Galloway et al. supra; Ikenberg et al. (1983) Int. J. Cancer 32:563-565). In these disorders, certain viral E genes and their protein products have exhibited prominent roles. Experiments using a reporter gene such as chloramphenicol acetyl transferase have shown that the E6 gene transactivates the noncoding region of HPV DNA. (Phelps et al. (1988) Cell 53:539-547). The E7 nucleoprotein has demonstrated a role in transformation and maintenance of malignant phenotypes in mammalian cells. (Tsunokawa et

al. (1986) Prooc. Natl. Acad. Sci. USA 83:2200-2203; Kanada et al. (1988) J. Virol. 62:610-613; Crook et al. (1989) EMBO J. 8:513-519).

Studies in experimental animals, usually mice, have shown that immunization with living or killed cancer cells can lead to rejection of a subsequent challenge by viable cancer cells. In many cases the target antigens responsible for the protective effects have been virally encoded, but in many other cases the nature of the antigen which elicits a protective immune response is unknown.

A major theoretical objection to the proposed use of cancer vaccines in humans is that humans who are "vaccinated", for example, with killed cancer cells or cell-free preparations, can be immunologically unresponsive. This is believed to often occur because the tumor antigens that may be the targets of the immune response are present, albeit in trace amounts only, in some normal cells and will thus be perceived by the immune system as "self". Immunization against such "self" antigens could, if effective, result in an autoimmune response. Most, if not all, tumor-associated antigens detected in human tumors by monoclonal antibodies are also present in some normal tissues, and there is little evidence that cancer patients respond to them effectively in vivo. An antigen that is foreign to the human immune system, for example, one encoded by an oncogenic virus such as HPV16, should, on the other hand, most likely induce a strong immune response.

The use of recombinant DNA technology for the production of vaccines to protect against viral infections and cellular transformation involves the molecular cloning and expression in an appropriate vector of genetic information coding for viral proteins which can elicit an immune response against the protein in the host animal. A novel approach has been described which is potentially useful in the production of such vaccines. (Mackett et al.

(1982) Proc.:. Natl. Acad. Sci. 79:7415-7419; Mackett et al. (1984) J. Virol. 49:857-864; ; Panicali et al. (1982), Proc. Natl. Acad. Sci. 79:4927-4931). This approach invvolves the use of vaccinia virus as a vector to express foreign genes inserted into ɔ its genome. Upon introduction into host animals, the recombinant vaccinia virurus expresses the inserted foreign gene and thereby elicits a host immune respponse to such gene products. Since live recombinant vaccinia virus can be used a as a vaccine, this approach combines the advantages of both subunit and live vaccicines.

Recommbinant vaccinia viruses expressing antigens from foreign viruses have been foound to induce resistance to challenge with the foreign viruses in experimental d animals. Examples include recombinant vaccinia viruses expressing aran HSV glycoprotein (Cremer et al. (1985) Science 228:1985), a rabies virus s surface antigen (Blancou et al. (1986) Nature 322:373), recombinant : vaccinia virus expressing either HPV16 or bovine papilloma virus proteins (Lathe et al. (1989) in Vaccines for Sexually Transmitted Disease, A. Meheus and I R. E. Spiel, eds, Butterworth & Co. pp. 166-177) and an influenza virus nucleopprotein (Smith et al. (1983) Proc. Natl. Acad. Sci. USA 80:7155; Yewdell et alal. (1985) Proc. Natl. Acad. Sci. USA 82:1785). The recombinant vaccinia viruus expressing influenza virus nucleoprotein has been reported to induce specifific T cell-mediated immunity to influenza virus in immunized mice (Bennink et alal. (1984) Nature 311:578). In addition, using target cells infected with a recommbinant vaccinia virus expressing influenza virus nucleoprotein, it has been deremonstrated that influenza virus nucleoprotein is recognized by cytotoxic T cells from influenza seropositive donors (McMichael et al. (1986) J. Gen. Virol. 67:719). Similarly, it has recently been found that human target cells infecteed with a recombinant vaccinia virus expressing an HSV

glycoprotein, a are recognized by human CTL clones specific for HSV (Zarling et al. (1986) J. VVirol. 59:506).

## SUMMARY OF THE INVENTION

The ppresent invention is directed to recombinant cells, peptides, antibodies, compositions, and methods for the inhibition and treatment of human papillomma virus infection and tumor initiation or progression.

The recombinant cells of the present invention contain a gene encoding a peptide that suubstantially corresponds to an amino acid residue sequence of a peptide expresssed in response to a human papilloma virus infection, such as a peptide substaantially corresponding to a region of the E6 and/or E7 gene product or a chimeric peptide compound of one or more regions of HPV proteins. Thihis peptide can substantially correspond to an HPV protein expressed uponn HPV infection or to a cellular peptide expressed in response to insertion of an HPV gene into the mammalian cell. Recombinant cells of the present inventidion include both eukaryotic and prokaryotic cells transfected or transformed, rrespectively, by the incorporation of added DNA encoding a region of a prrotein of human papilloma virus. Illustrative recombinant cells include bacteriia, viruses, such as vaccinia virus, mammalian cells such as transfected epitlithelial or fibroblast cells or lymphoid cells and tumor cells that encode such ann HPV related protein region, such as cervical carcinoma cells. Soluble proteinss and peptides that elicit B cell and/or T cell responses are also included in the p present invention.

Antibodyly molecules that can mimic and/or compete for binding sites with such proteteins and peptides are also included in the present invention. Particularly preeferred antibodies are antibodies to peptides corresponding to

specific regionns of the HPV16 E6 and/or E7 proteins and anti-idiotypic antibodies to thhese anti-peptide antibodies.

Compossitions of the present invention contain recombinant cells, antibodies and/l/or peptides as described above, and preferably recombinant cells and/or peptidess which express an epitopic region of an E6 or E7 nucleoprotein of human papipilloma virus. The compositions of the present invention are preferably imnmunogenic compositions that are capable of eliciting an immunologicallilly protective response in a recipient.

The dessscribed recombinant cells, peptides, and compositions are utilized in methods for r inhibiting and treating HPV infection and tumor initiation and/or progression. ] In a method of the present invention for the treatment of a condition resulltant from human papilloma virus infection, a therapeutically effective amounnt of a composition containing a recombinant cell and/or peptide of the present i invention is administered to a patient for a time period sufficient to inhibit the fururther progression of this condition.

A propphylactic method for inhibiting tumor initiation following the detection of huuman papilloma virus infection is further contemplated. In this method, a therrapeutically effective amount of a composition of the present invention is adrministered to a patient in order to elicit a protective response in that patient thatat inhibits tumor initiation in the virus-infected cells. The present invention is fururther directed to a method of inhibiting human papilloma virus infection in a ppatient. In this method a sufficient amount of an immunogenic composition is s administered to a patient to effectively elicit an immunologically protective respoonse in the patient to inhibit infection by human papilloma virus.

The immmunogenic composition can also be formulated to contain recombinant ceells that express an epitopic region of an HPV protein. For example, such ı compositions can contain a non-tumorigenic cell that is major

histocompatibilility complex (MHC) class I positive, into which a gene encoding an immunogeniaic region of an HPV protein has been inserted. The recombinant cell of this commposition can then be administered to a patient to facilitate tumor rejection by eliciciting an immunogenic response to the expressed peptide region, which is also exxpressed in the tumor cells.

The immmunogenic composition can also be formulated as a viral vaccine, in which case tlthe immunogen comprises a recombinant virus that expresses an epitopic region ( of a protein of the human papilloma virus. Depending upon the nature of the rerecombinant virus used as the immunogen, either an inactivated virus vaccine ( or a live virus vaccine can be formulated. Appropriate immunization wwith the vaccine formulation or immunogenic composition of the present inventioon can result in the induction of an immune response which leads to the destructiion of tumor cells expressing an HPV epitopic region as well as inhibiting HPV / infection, in the immunized subject. Preferred recombinant cells of the presesent invention include vaccinia virus encoding and expressing an epitopic region ( of either the E6 or E7 HPV16 nucleoprotein, and epithelial, fibroblast, lymphhoid, blood cells, and tumor cells transfected with the E6 or E7 HPV16 gene.

Still furthther advantages and benefits of the present invention will become apparent to thoose skilled in the art in the following detailed description, examples and clalaims which follow.

## DESCRIPTION OF DRAWINGS

In the drawings: ::

**FIGUREE 1** illustrates the cloning of the HPV16 E6 open reading frame into two expressision vectors.

A. The E6 open reading frame (ORF) was removed from HPV16 full length DDNA and cloned as a blunt-ended DdeI fragment into vaccinia expression veiector pGS 62 which had been cut with SmaI.

B. The E6 ORF was cloned in pIC 20H to introduce a Hind III site at the 5' end i for directional cloning downstream of the CMV promoter in the pCDM8 expiression plasmid as described hereinbelow. There are 58 base pairs at the 5' and ç 98 base pairs at the 3' end of the E6 ORF which are untranslated.

FIGUIRE 2 illustrates the cloning of the HPV16 E7 open reading frame into two expiression vectors.

A. Thhe E7 open reading frame was cloned from the full length HPV16 DNA as a TaiaqI, PstI fragment into pIC 20R which had been cut with ClaI and PstI. The E77 ORF was subcloned into the vaccinia expression vector pGS 62 at the EcoRI sitete.

B. Thhe E7 ORF was cloned into the pIC 20H vector to introduce a Hind III site at the ; 5' end of the gene and was placed under the control of the CMV promoter in i the pCDM8 vector. There are 56 base pairs of untranslated sequence 5' tato the E7 ORF and 24 base pairs 3' to the E7 ORF.

FIGUIRE 3 illustrates the autoradiography of radioimmunoprecipitations of lysates froom cells infected by two different plaque purified recombinant vaccinia virusses expressing the E6 protein of HPV16.

The vvaccinia lysates were prepared as described in EXAMPLE 5. Radioimmunopprecipitations were performed and the precipitates were electrophoresesed. Lanes 1, 3, and 5 show the results obtained using rabbit antisera to E66 (provided by D. Lowy), lanes 2, 4, and 7 with normal rabbit serum and lanne 6 with rabbit antisera to E7 (α16 E7 NP). The antigens are

noted above thete lane numbers. The lysates were prepared from labelled infected cells, and the elelectrophoresis gel was 17.5% polyacrylamide.

FIGURRE 4 illustrates the autoradiography of recombinant vaccinia lysates precipititated with rabbit antisera against either the E6 or the E7 nucleoproteins c of HPV16.

The vacccinia recombinant-infected cells were labelled for one hour with $^{35}$S-Cys and $^{35;5}$S-Met. The infecting virus is noted above the lane numbers. The radioimmuunoprecipitates were loaded such that lanes 1 and 5 show the results obtained d with anti-HPV16 E6 rabbit serum (D. Lowy), lanes 2, 4 and 7 show the resultsts obtained with normal rabbit serum; and lanes 3 and 6 show the results obtained d with α16 E7 NP. The position of standard stained molecular weight markers s are noted on the right side, while the position of E6 and E7 are noted by the leftft arrows.

FIGUREE 5 illustrates a pulse-chase study of the stability of the E7 protein.

Vaccinia a virus infected cells were pulse labelled for 1 hour as described hereinbelow witlith $^{35}$S-Cys and $^{35}$S-Met, then incubated with unlabelled medium for the time peririods shown above the lane numbers. The infecting virus (vNY or vHPV16/E7)') is listed above the time identifications. After the indicated periods of timme, the cells were lysed and maintained at 0-4°C for radioimmunopreecipitation analysis on a 17.5% acrylamide gel. The odd numbered lanes:s represent α16 E7 NP precipitation products, while the even-numbered lanes represent normal rabbit serum immunoprecipitation products. Molecccular weight markers are indicated on the right.

FIGUURE 6 illustrates radioimmunoprecipitation of the E7 gene product expressed in ı COS cells transiently transfected with pCDM8-E7 mammalian expression plalasmid.

Lanes s 1 and 3 show the banding pattern obtained for proteins precipitated wwith rabbit anti-Trp E/E7.

Lanes s 2 and 4 show the banding pattern obtained for proteins precipitated wwith normal rabbit serum.

N = NNuclear fraction.

C = CCytosol fraction.

A. VVaccinia E7 recombinant lysate was used as positive control for E7 protein.

B. pCCDM8 E7 transfected COS cells.

C. UnUntransfected COS cells.

FIGUURE 7 illustrates the amino acid residue sequences for the HPV16 E6 and E7 nuucleoproteins

FIGUURE 8 illustrates the titration of two monoclonal antibodies against peptide 359 obf the HPV16 E7 protein. The open squares show hybridoma clone #14, the closeed squares show hybridoma clone #10.

FIGUURE 9 illustrates Western blots of the titration of antisera against E6 peptides. Antıti-E6 peptide sera taken 3 days post-boost from rabbits immunized with peptide : from the E6 ORF were titrated on Western blots against the

homologous specific Trp fusion protein (16 E6 DS) only. The numbers (1) and (2) indicate two different rabbits immunized with the same peptide.

FIGURE 10 illustrates Western blots of the titration of antisera against E7 peptides. Anti-E7 sera taken 3 day post-boost from animals immunized with peptides from the E7 ORF were titrated on Western blots against the specific Trp fusion protein (16 E7 NP). The numbers (1) and (2) indicate two different rabbits immunized with the same peptides.

FIGURE 11 illustrates Western blots of two dilutions of antisera against E6 and E7 peptides. Sera from rabbits immunized with an E6 or E7 peptide were tested by Western blotting against the specific Trp fusion protein and the Trp E vector gene product to demonstrate that the reactivity in the serum was specific for the HPV16 E6 or E7 protein. The highest serum dilutions were selected to be near the endpoint of reactivities based upon previous titrations.

The specific peptide (by number) used as the immunogen is listed above each set of nitrocellulose lanes; the antigen in each lane is denoted at the top of the gel and the two dilutions of the serum used are shown at the bottom under the brackets. M indicates prestained molecular weight markers. Serum samples were drawn about 1 week after the first or second boost for use in these studies. Antisera α358, α360 and α361 were obtained one week after the second boost. Antiserum α359 was obtained one week following the first boost.

FIGURE 12 illustrates the recognition of E7 native protein by anti-E7 peptide antisera. Anti-peptide 359 antisera from rabbits bled one week after the first boost were used in a radioimmunoprecipitation assay on $^{35}$S-methionine

and $^{35}$S-cysteine labelled vaccinia E7 recombinant-infected cell lysates. The control α16 ] E7 NP (lane 1) precipitates a band of 17-18 kDa, the rabbit sera (lanes 3 and 4) similarly precipitate a band of the same molecular weight, while the α360 serum (Lane 2) fails to precipitate an E7 band. Molecular weight markers are s seen at the right.

**FIGUURE 13** illustrates RT-PCR analysis of cytoplasmic RNA from HPV16 E7 transfectants. Lanes contain (from left to right) pCDM8/E7 plasmid, as a positive PCR control; cytoplasmic RNA from N7.4 and N7.2 cells, respectively, which are NCTC 2555-derived HPV16 E7 transfectants; NCTC 25555 cells, as a negative control; E7C3, which is an M2 melanoma-delerived HPV16 E7 transfectant; and M2 melanoma cells as a negative contntrol (par). Standard base pair markers are seen on the left.

**FIGUURE 14** illustrates the growth of E7C3 tumors in C3H/HeN mice. In panel A, mice were immunized prior to tumor cell inoculation with NCTC 2555 fibroblalasts that have been transfected with either the HPV16 E7 (N7.2 and N7.4, open triangles, and closed circles, respectively) or HPV16 E6 (N6.8, open circles)) gene. Panel B illustrates control studies that were carried out by inoculating mmice with either phosphate buffered saline (PBS, open squares) or NCTC 2555 fibroblasts transfected with human melanoma antigen p97 (CL19, closed trianglgles), relative to immunization of the mice with N7.2 cells prior to E7C3 tumor cell inoculation. Panel C illustrates the tumor growth of the parental M2 melanoma cells (par) in mice immunized with E7-transfected NCTC 2555 fibroblasts (N 7.2).

FIGURRE 15 illustrates the effect of anti-CD8 antibody treatment on tumor growth in mice immunized with N7.2 cells. Following immunization, mice were injected with either anti-CD8 antibodies (closed circles) or anti-CD5 antibodies (open triangles) prior to administration of E7C3 tumor cells.

FIGURRE 16 illustrates a flow cytometric analysis of CD4 positive and CD8 positive splenocytes from mice treated with anti-CD8 monoclonal antibody (right panels) or with anti-CD5 monoclonal antibody (left panels).

## DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to recombinant cells, peptides, antibodies, compositions, and methods that are useful for the inhibition and treatment of human papilloma virus infection and tumor initiation. The recombinant cells contain a DNA construct encoding a region of an HPV protein, preferably E6 and/or E7 HPV nucleoprotein. These recombinant cells express an epitopic region of a peptide substantially corresponding to a peptide expressed in a mammalian cell in response to the insertion of an HPV gene into the mammalian cell, such as by HPV infection or recombinant means. In a preferred embodiment, peptides of the present invention substantially correspond to about 8 to about 30 amino acid residue regions of HPV protein. In a particularly preferred embodiment the peptides of the present invention substantially correspond to a region of the HPV16 E6 or E7 protein, and specifically to an epitopic region capable of eliciting an immunological interaction with an antibody and/or T-cell surface molecule when administered to a host. The present invention also encompasses compositions of these recombinant cells, and peptides that can be utilized as immunogenic

compositions, , immunotherapeutics or vaccines for treatment of patients. Antibody mololecules that can compete for binding sites with these peptides are also contemplplated herein. Specifically, antibody molecules against binding regions of thee HPV16 E6 and/or E7 proteins, and anti-idiotypic antibodies against these ; antibodies, are produced that compete for HPV binding sites on normal and t tumor cell proteins, such as the retinoblastoma gene product (RB105) whicch binds to the HPV16 E7 protein. The present invention is further directed to mmethods of inhibition and treatment of human papilloma virus infection and l oncogenesis. Specific methods of the present invention are directed to thee treatment of a condition resultant from HPV infection, and to a prophylactic r method to inhibit tumor initiation and progression from cells following the : detection of HPV infection. A further aspect of the present invention is diirected to a method of inhibiting HPV infection in a patient.

I. Definitions

In ordder to more clearly describe the present invention and its embodiments, , the following definitions are included.

"Transfsfection", as used herein, is the acquisition of new genetic markers by incorporaticion of added DNA into eukaryotic cells.

"Transfsformation", as used herein, is the acquisition of new genetic markers by incacorporation of added DNA into prokaryotic cells.

"Oncoggenesis", as used herein, is the cellular acquisition of a neoplastic phenotype leadding to uncontrolled cell proliferation.

"Clonining vector", as used herein, is any plasmid or virus into which a foreign DNA r may be inserted to be cloned.

"Plasminid", as used herein, is an autonomous self-replicating extra-chromosomal c circular DNA.

"Open Reading Frame" (ORF), as used herein, is a DNA sequence which is (potentially) translatable into protein.

"Helper virus", as used herein, is a virus that provides functions absent from a defective virus, enabling the latter to complete the infective cycle during a mixed infection.

"Gene (cistron)", as used herein, is the segment of DNA that encodes the sequence of a peptide chain; it can include regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

"Expression", as used herein, is the process undergone by a structural gene to produce a peptide or protein. It is a combination of transcription and translation.

As used herein, the term "clone" describes any number of identical cells or molecules with a single ancestral cell or molecule.

As used herein, the term "base pair" (bp) is a partnership of adenine (A) with thymine (T), or of cytosine (C) with guanine (G) in a DNA double helix.

As used herein, the term "expression vector" is any plasmid or virus into which a foreign DNA may be inserted and/or expressed.

As used herein, the term "downstream" identifies sequences proceeding further in the direction of expression; for example, the peptide coding region of a gene is downstream from the initiation codon or in the 3' direction away from the gene; upstream is 5' to the sequence in question.

As used herein, the term "polymerase chain reaction" (PCR) refers to the amplification of DNA molecules by the successive use of a temperature stable DNA polymerase to copy the DNA chain, separating the complementary chains by heating, adding primers and repeating the process about 30 times to produce approximately $10^9$ copies of the DNA. By use of the PCR technique,

minute amounnts of DNA can be amplified to produce sufficient DNA for use in various proceedures.

The tterm "inoculum" in its various grammatical forms is used herein to describe a coomposition containing a recombinant cell or peptide of this invention as an active i ingredient used for the preparation of antibodies or elicitation of T cells against h human papilloma virus infected/transformed cells. When a peptide is used to ininduce antibodies it is to be understood that the peptide may be infrequently r used alone, and more often linked to a carrier or in combination with other cicomponents but for ease of expression these alternatives will not always be exxpressed hereinafter.

Inoculula may also include an adjuvant. Adjuvants such as complete Freund's adjjjuvant (CFA), incomplete Freund's adjuvant (IFA) and alum are materials welell known in the art and are available commercially from several sources.

Individividual inocula are readily prepared with CFA or IFA. For example, an amount o of recombinant cell or peptide conjugate sufficient to provide the desired amoount of recombinant cells and/or peptide conjugate per inoculation is dissolved in l PBS (at about 0.5 ml) at pH 7.2. Equal volumes of CFA or IFA are then miixed with the solution to provide an inoculum containing the recombinant t cells and/or conjugate, water and adjuvant in which the water to oil ratio is 1:1. . The mixture is thereafter homogenized to provide the inoculum. The volume s of the inoculum so prepared is typically greater than 1 ml, and some of the r recombinant cells and/or peptide conjugate, PBS and adjuvant may be lost duringg the emulsification. Substantially all of the emulsification that can be recovered d is placed into a syringe, and is then introduced into the animals as discussed herreinbelow. The amount of inocula introduced into an animal, such

as a rabbit or mouse, should be at least about 90% of that present prior to the emulsification step.

In inocula of the present invention both recombinant cells and peptides may be included either alone or conjugated to a carrier protein such as keyhole limpet hemocyanin (KLH) plus a physiologically acceptable diluent such as water or PBS along with an adjuvant. KLH is an acceptable carrier for use in animals, but it is quite costly to use on a commercial scale. The use of alternative carriers including soybean agglutinin, aluminum hydroxide (alum), bovine serum albumin (BSA), ovalbumin, peanut agglutinin, tetanus toxoid and poly-L-lysine is also contemplated. Saponin, a plant produced glycoside, is also a well known adjuvant available commercially from Berghausen Chemical Company, Cincinnati, Ohio, as a 5% solid solution, and can be used herein along with aluminum hydroxide.

The above inocula stock solutions are illustrative of the inocula of this invention. As demonstrated herein, they can be used to produce antibody molecules or elicit T cells that immunoreact with the recombinant cells and/or peptides of the present invention.

The term "immunogenic composition" in its various grammatical forms is used herein to describe a type of inoculum containing a recombinant cell or peptide of this invention, that is, an active ingredient that is used to induce an active immunity in a host animal. In a preferred embodiment, an immunogenic composition of the present invention can be a vaccine. Since active immunity involves both the production of antibodies and the elicitation of a cell-mediated immune response, a vaccine and an inoculum may thus contain identical ingredients, but their uses are different. In most cases, the ingredients for a vaccine and for an inoculum are different because many adjuvants used for animals may not be used in humans.

The relatively small peptides used in the studies of the present invention were synthesisized using the solid-phase method of Merrifield, (1963) J. Am. Chem. Soc. 85:2149-2154, incorporated herein by reference, on an Applied Biosystems Peptide Synthesizer, Model 430A. An additional cysteine residue was inserted into the sequence at either the C- or N-terminus. After cleavage from the resin and deprotection, the peptides were purified by reversed-phase high performance liquid chromatography. Prior to their use as immunogens the peptides were coupled, through their cysteine residues, to KLH by use of the bifunctional reagent sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate.

The term "synthetic" as used herein refers to a peptide molecule that has been built up by chemical means, that is, chemically synthesized, rather than being prepared by a biological means such as by genetic engineering techniques.

Vaccines and immunogenic compositions used herein contain the stated amount of peptide alone, recombinant cells, conjugates or combinations thereof. These immunogenic compositions also contained a physiologically tolerable diluent such as water or saline, further typically including an adjuvant, such as complete Freund's adjuvant and incomplete Freund's adjuvant.

Immunogenic stock solutions were prepared with IFA or CFA as follows: An amount of the synthetic peptide conjugate and/or recombinant cells sufficient to produce the desired amount per inoculation was dissolved in phosphate buffered saline (PBS). Equal volumes of CFA or IFA were then mixed with the solution to provide a composition containing the cells and/or peptides, water and adjuvant in which the aqueous-to-oil ratio was 1:1. The mix was thereafter homogenized to provide the stock solution.

As used herein, an "epitopic region" is a structural domain, such as a specific amino acid residue sequence or peptide fragment, of a molecule that is

capable of eliciting a specific immunological interaction with antibody molecules or T cell surface molecules in a host. An epitopic region can contain one or more antigenic determinants and/or immunogenic determinants.

The term "antigenic determinant" as used herein, designates the structural component of a molecule that is responsible for specific interaction with corresponding antibody (immunoglobulin) molecules or T cell surface molecules elicited by the same or related antigen or immunogen.

The term "immunogenic determinant", as used herein, designates the structural component of a molecule that is responsible for the induction in a host of an antibody or T cell surface molecule containing an antigen combining site (idiotype) that binds with an immunogen when used as an antigen.

The terms "anti-idiotype" and "anti-idiotypic antibody" are used interchangeably herein, and refer to an antibody whose antigen binding site specifically binds to the idiotype of the primary antibody prepared against a particular antigen, such as a papilloma virus antigen, such that the anti-idiotypic antibody competes for the binding of the primary antibody to the antigen.

The term "antigen", as used herein, refers to an entity that is bound by an antibody or a T cell surface molecule which develops in response to the presented structural component.

The term "immunogen", as used herein, describes an entity that induces antibody or specific T cell production responses in the host animal.

The term "unit dose" refers to physically discrete units suitable as unitary dosages for animals, each unit contains a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; that is, a carrier or vehicle. The specifications for the novel unit dose of this invention are dictated by, and are directly dependent upon, (a) the unique characteristics of the active material and the particular

therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such active material for therapeutic use.

As used herein the term "effective amount" means an amount sufficient to beneficially inhibit the infection and/or tumor initiation of cells in response to an HPV infection. The effective amount for a particular patient may vary depending on such factors as the state of the infection, the overall health of the patient, the method of administration, the severity of side effects, and the like.

The term "correspond" in its various grammatical forms, as used herein and in the claims in relation to peptide sequences means the peptide sequence described plus or minus up to 3 amino acid residues at either or both of the amino and carboxy termini and containing only conservative substitutions in particular amino acid residues along the peptide and/or polypeptide sequence.

The term "conservative substitution" as used above denotes that one amino acid residue has been replaced by another, biologically similar residue. Examples of conservative substitutions include the substitutions of one hydrophobic residue such as Ile, Val, Leu, or Met for another, or the substitution of one polar residue for another such as between Arg and Lys, between Glu and Asp or between Gln and Asn, and the like.

In some instances the replacement of an ionic residue by an oppositely charged ionic residue such as Asp by Lys has been determined conservative in the art in that those ionic groups are thought to merely provide solubility assistance. In general, however, since the replacements discussed herein are on a relatively short synthetic peptide region, as compared to a whole protein, replacement of an ionic residue by another ionic residue of opposite charge is considered herein to be a "radical replacement" as are replacements by nonionic and ionic residues, and bulky residues such as Phe, Tyr or Trp and less bulky residues such as Gly, Ile and Val.

The terms "nonionic" and "ionic" residues are used herein in their usual sense to designate those amino acid residues that either bear no charge or normally bear a charge, respectively, at physiological pH value. Exemplary nonionic residues include Thr and Gln, while exemplary ionic residues include Arg and Asp.

II. Description of Preferred Embodiments

The inhibition and treatment of human papilloma virus infection, oncogenesis and tumor initiation in patients are aims of the present invention. The demonstration of the expression of papilloma proteins and peptides in infected cells has encouraged the present inventors to develop the recombinant cells, peptides and methods described herein.

Recombinant cells of the present invention contain a gene insert that is substantially similar to a DNA region from a human papilloma virus. In one embodiment, the gene insert encodes a region of the E6 and/or E7 nucleoprotein of HPV16. In a particularly preferred embodiment, an epitopic region of the E6 and/or E7 protein is expressed. In another embodiment, the gene insert induces the expression of a cellular protein in a recombinant mammalian cell containing this gene insert.

The present invention contemplates expression systems for HPV-induced protein regions and include viruses such as vaccinia virus and adenovirus, fibroblasts such as COS monkey cells and human keratinocytes, tumor cells such as CaSki cervical carcinoma cells, and melanoma cells, and other mammalian cells such as epithelial cells, and lymphoid cells. In another embodiment, cells which are MHC class I positive, and which preferably are non-tumorigenic, are transfected with a gene encoding a region of an HPV protein. In a particularly preferred embodiment, a gene encoding a region of

the HPV16 E6 and/or E7 protein is inserted into these MHC class 1 positive cells or is expressed upon infection with a recombinant virus containing these genes, and an immunogenic composition containing these cells are administered to a patient having an HPV16 induced tumor as a method of facilitating tumor regression. Such tumor regression can be in response to the induction of an immunological response in the patient by the immunogenic cells and this response is then directed against the tumor.

The recombinant cells of the present invention are expression systems, or cells, that contain an inserted gene construct encoding a region of an HPV protein. Illustrative expression systems, or cells, in the present invention include viruses such as vaccinia virus, amphitropic retroviruses, adenovirus, poliovirus and other viruses that can be administered to patients in a non-pathogenic manner, such as in an immunogenic composition or a vaccine, and cells capable of infection, transfection or transformation with an HPV gene such as peripheral blood lymphocytes, and epithelial cells. The present invention contemplates all cells that are capable of integrating and expressing a region of a human papilloma virus gene. Some variations in the amino acid residue sequence of the expressed gene product that do not significantly diminish immunogenicity are contemplated in this invention, as well as specific peptide fragments having similar amino acid residue sequences to region of E6 and E7.

In one embodiment the gene constructs of the present invention are prepared by cloning an ORF corresponding to a region of a human papilloma virus nucleoprotein, such as the E6 or E7 proteins of HPV16, from a plasmid containing a larger portion of the HPV16 genome by restriction endonuclease. The restriction fragments are then cloned into expression vectors by standard molecular biology procedures, such as those described in Ausubel, F. M. et al.

(1990) Current Protocols in Molecular Biology (Greene Publishing Assoc. and Wiley Interscience) and Maniatis, T. et al. (1982) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, NY).

The expression vector containing the desired ORF is then further processed and inserted into the genome of a host cell to produce the recombinant cell of the present invention, such as by homologous recombination or integration forced by selective pressure; or introduced by viral infection of the cells.

The particular site chosen for insertion of the selected ORF fragment into the cloning vehicle to form a recombinant DNA molecule is determined by a variety of factors, known by one skilled in the art, such as size and structure of the peptide or protein to be expressed, susceptibility to degradation of the gene product by the host cell and location of standard stop codons.

In a particularly preferred embodiment, a host cell of the present invention is a vaccinia virus. Vaccinia virus contains a linear double-stranded DNA genome of approximately 187 kilobase pairs and replicates within the cytoplasm of infected cells. These viruses contain a complete transcriptional enzyme system (including capping, methylating and polyadenylating enzymes) within the virus s core that are necessary for virus infectivity. Vaccinia virus transcriptional regulatory sequences (promoters) allow for initiation of transcription by vaccinia RNA polymerase but not by host cell RNA polymerase.

Expression of foreign DNA in recombinant vaccinia viruses requires the ligation of vaccinia promoters to protein-coding DNA sequences of the foreign gene. Plasmid vectors, also called insertion vectors, have been constructed to insert foreign genes into vaccinia virus. One type of insertion vector is composed of: (a) a vaccinia virus promoter including the transcriptional

initiation site; ; (b) several unique restriction endonuclease cloning sites located downstream frfrom the transcriptional start site for insertion of foreign DNA fragments; (c) ) nonessential vaccinia virus DNA (such as the TK gene) flanking the promoter aand cloning sites which direct insertion of the foreign gene into the homologous nconessential region of the virus genome; and (d) a bacterial origin of replication a and antibiotic resistance marker for replication and selection in E. coli. Examples of such vectors are described by Mackett (Mackett et al. 1984, J. Virol. 49:85'57-864).

Recombinant vaccinia viruses are produced after recombinant bacterial insertion plasmids, containing the foreign gene, are transfected into cells previously infected with vaccinia virus. Homologous recombination takes place within the infected cells and results in the insertion of a foreign gene into the viral genome. The infected cells can be screened using immunological techniques, DNA plaque hybridization, or genetic selection for recombinant viruses which subsequently can be isolated. These vaccinia recombinants retain their essential functions and infectivity and can be constructed to accommodate approximately 35 kilobases of foreign DNA. Foreign gene expression can be detected by examining RNA levels using Northern blotting or dot blotting and nucleic acid hybridization or by examining protein levels using immunological assays (for example, radioimmunoprecipitation, radioimmunoassay, or immunoblotting).

Peptides and peptide-conjugates of the present invention contain amino acid residue sequences that substantially correspond to regions of expressed HPV proteins. The peptides preferably correspond to those regions of HPV-induced proteins expressed in the recombinant cells of the present invention. Particularly preferred peptides correspond to epitopic regions of HPV 16 E6 and/or E7 proteins. The peptides can be prepared by either the

solid phase synnthesis method of Merrifield, referred to above, or by standard genetic engineeering methodology.

Composisitions containing recombinant cells and/or peptides of the present invention are uutilized as immunogenic compositions, vaccines and therapeutic compositions. In one embodiment, a composition containing a recombinant vaccinia virus t that expresses an epitopic region of the HPV16 E7 protein is utilized as an immmunogenic composition that can elicit a protective response in a patient to HPV *i* infection and/or tumor initiation.

The commpositions of the present invention contain, in addition to the recombinant ceells or peptides described herein, a physiologically tolerable diluent such as s water or saline, and further typically include an adjuvant, as described hereinin above.

The presesent invention also involves administering an effective amount of the recombinantnt cells and/or peptides, preferably expressing an HPV epitopic region, to a patitient suffering from a condition resultant from HPV infection.

Generallyly, the recombinant cells and/or peptides of the present invention are administereed as a pharmaceutical composition comprising an effective amount of the rrecombinant cells and/or peptides and a pharmaceutical carrier. When administetered parenterally, the composition of the present invention is formulated in a ı unit dosage injectable form (typically a solution, suspension or emulsion) in asıssociation with a pharmaceutical carrier. Such carriers are inherently non-tctoxic and non-therapeutic. Examples of such carriers are normal saline, Ringer's s solution, dextrose solution and Hank's solution. Nonaqueous carriers such as s fixed oils and ethyl oleate may also be used. A preferred carrier is 5% d dextrose/saline. The carrier can contain minor amounts of additives such a as substances that enhance immunogenicity, isotonicity, and

chemical stability, for example, buffers and preservatives. Generally, carriers useful for such administration are well known in the art.

Antibodies and substantially whole antibodies raised to (induced by) recombinant cells and peptides of this invention as well as antigen combining sites prepared from such antibodies and anti-idiotypic antibodies prepared to these antibodies and/or antibody fragments constitute still another embodiment of this invention. Such antibodies are raised in mammalian hosts such as mice, rats, guinea pigs, rabbits, horses and the like by immunization using the inocula described hereinabove, or monoclonal antibodies conjugated to carriers for the purpose of raising anti-idiotypic antibodies.

In a preferred embodiment, the antibody molecules of this invention include whole antibody raised in mammals by immunizing them with inoculum containing a recombinant cell and/or peptide or anti-peptide antibody as described hereinabove.

Antibodies prepared against specific peptides of E6 and E7 will allow the examination of cellular homologs of E6 and E7. For example, peptides. such as peptide 359, contain part of a binding region for a retinoblastoma gene product termed RB105. Antibodies against such peptides may mimic RB105 and bind to cellular proteins sharing sequence homology with E7. This identification of cellular homologs of E7 could potentially identify cellular proteins responsible for proliferation, and cellular proteins which may be normal ligands of RB105. The anti-idiotypes of such antibodies may mimic E7 and identify ligands other than RB105 of E7 itself or the homologs, i.e., cell proliferation proteins. This can lead to identification of other cellular proteins which interact with cellular proliferation proteins to either up or down-regulate proliferation, such as tumor suppressor proteins or transforming proteins. Similar studies can be applied to E6 and its ligand p53 and other unknown ligands of either E6 or p53.

Rabbits may be immunized with inocula containing 50 μg to 1.0 mg of recombinant cells and/or a peptide conjugate in complete Freund's adjuvant, and boosted two or three weeks later with 10 μg to 1.0 mg of recombinant cells or conjugated peptide in incomplete Freund's adjuvant. Each rabbit immunization consists of ten intradermal injections on the back, two of which are in the sub-scapular region and boosts in five sites, one being in the sub-scapular region. Rabbits were bled two weeks post-primary and one week (5 - 8 days) subsequent to the boosts.

Sera containing immunologically active antibodies would then be produced from the bleeds by methods well known in the art. These antibodies are immunoreactive with one or more of the peptides of this invention. Such antibodies can then be utilized by similar methodology to produce anti-idiotypic antibodies of one or more peptides of this invention.

Suitable monoclonal antibodies, typically whole antibodies, can also be prepared using hybridoma technology described by Niman et al. (1983) Proc. Natl. Acad. of Sci., USA 80:4949, which description is incorporated herein by reference. Briefly, to form the hybridoma from which the monoclonal antibody is produced, a myeloma or other self-perpetuating cell line is fused with lymphocytes obtained from the spleen of a mammal hyperimmunized with a recombinant cell or peptide or antibody of the present invention.

It is prefeferred that the myeloma cell line be from the same species as the lymphocytes, but cross-species hybrids can be raised in nude mice. Typically, a mouse of the strain Balb/c is the preferred mammal. Suitable mouse myelomas for use in the present invention include AG-8 cells and NS-1 cells.

Splenocytes are typically fused with myeloma cells using a polyethylene glycol, such as PEG 1500 or PEG 6000. Fused hybrids are selected by their sensitivity to HAT media (hypoxanthine, aminopterin, and thymidine).

Hybridomas ] producing the antibody molecules of the present invention are identified usiring the enzyme linked immunoabsorbant assay (ELISA) described hereinafter.

Monooclonal antibodies need not only be obtained from hybridoma supernatant, t but may also be obtained in generally more concentrated form, from ascites : fluid of mammals into which the desired hybridoma have been introduced. : Production of monoclonal antibodies using ascites fluid is well known and wivill not be dealt with further herein.

Methoods are contemplated by the present invention for the inhibition and treatment of : HPV infection and conditions resultant from HPV infection in patients.

In oneie embodiment, a method for the treatment of a condition resultant from HPV inhfection includes the administration to a patient of a therapeutically effective amoiount of a composition of the present invention for a time period sufficient to ; inhibit the progression of the condition. Illustrative conditions include cervicical warts and human cervical carcinoma, in which treatment with the compositition of the present invention prevents or retards the further progression obf the condition in the patient.

A propphylactic method is further contemplated to inhibit tumor initiation in a patient fofollowing the detection of HPV infection by which a therapeutically effective amoiount of the composition of this invention is administered to the patient to elicicit a protective response that inhibits tumor initiation. Preferably, administratiomn of an immunogenic composition containing the recombinant cells and/or peptidides of the present invention elicits the recruitment of CD8+ T lymphocytes t that mediate the inhibition of tumor initiation in the patient.

A metethod for inhibiting HPV infection in a patient at risk for exposure to HPV is a also contemplated in the present invention. In this method a

sufficient amount of an immunogenic composition containing the recombinant cells and/or peptides of this invention is administered to a patient to effectively elicit an immunologically protective response in the patient to inhibit subsequent infection by HPV.

In a preferred embodiment, the immunogenic composition is a vaccine that when administered immunized the patient against HPV infection.

The present invention is further described by the following Examples which are intended to be illustrative and not limiting.

## EXAMPLE 1

### Preparation of the ORF Constructs of E6 and E7

A. E6

The E6 and E7 ORFS were cloned from a pBR322 plasmid (pBR322/HPV16) containing the entire HPV16 genome. The 630 bp DdeI fragment (bp## 25-654) contains the E6 ORF. The DdeI fragments were blunt-ended with the Klenow fragment of DNA polymerase and subjected to gel electrophoresis in 3% NuSieve genetic technology grade (GTG) (FMC Bioproducts, Rockland, ME) agarose. The 630 bp DdeI fragment was electrophoretically transferred to NA45 DEAE (Schleicher & Schuel, Keene, N.H) paper, and eluted in high salt NET buffer (1.0 M NaCl, 0.1 mM EDTA, 20 mM Tris pH 8.0) extracted at 65° C with phenol and then chloroform, precipitated with 2 volumes of 100% ethanol, and, then, the DNA pellet was resuspended in 1 Tris-EDTA (TE) buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA, pH 8.0).

The vaccinia expression vector utilized was pGS62 which is identical to pGS20 (Mackett et al. (1984) J. Virol. 49:857-864.) with the exception that an EcoRI site was deleted from the plasmid, leaving only one EcoR site

downstream of the SmaI site. This vector was linearized with SmaI, and dephosphorylated by treatment with calf intestinal alkaline phosphatase (CIAP) and gel purified. Recovery of the fragment from the gel was performed as described for the E6 ORF from pBR322. The E6 ORF, plus untranslated sequences of 58 bp 5' and 98 bp 3', was ligated to the pGS62 vaccinia expression vector downstream of the 7.5 k promoter. The recombinant plasmid, having the structure indicated in FIGURE 1, was isolated, characterized and propagated by standard molecular biological procedures as described in Maniatis, T. et al. (1982) in Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, New York) and Ausubel, F. M. et al. (1990) Current Protocols in Molecular Biology (Greene Publishing Assoc. and Wiley Interscicience).

B. E7

The complete HPV16 genome cloned in pBR322 was cleaved with TaqI and PstI and subjected to electrophoresis. The 374 bp fragment containing the E7 ORF was gel purified as described for E6. The pIC 20R vector (Marsh, J.L., et al. (1984) Gene 32:481-485) was cleaved with PstI and ClaI and gel purified. The recombinant pIC 20R E7 plasmid having the indicated structure (FIGURE 2) was isolated, characterized and propagated as described above. The pIC 20RR E7 was treated with EcoRI and the fragment containing the E7 ORF was purified by gel electrophoresis.

The vaccinia expression plasmid pGS62 was cleaved with EcoRI, treated with calf intestinal alkaline phosphatase and gel purified as described. The recombinant plasmid indicated in FIGURE 2 containing the E7 ORF and 56 bp

untranslated 5'5', and 24 bp 3' of the E7 ORF was obtained, characterized and propagated.

C.

Both rerecombinant vaccinia expression plasmids were expanded and purified by CsGCl, ethidium bromide equilibrium centrifugation.

## EXAMPLE 2

Construction of Recombinant Vaccinia Virus

The clooning steps for either E6 or E7 ORFs were designed to insert the open reading frframes at a unique cloning site just downstream of a vaccinia virus transcriptional control element (7.5 k promoter) which is expressed at both early and late times after infection (Earl et al, (1990) J. Virol. 64:2448-2451). The ORFs are flankked by the left and right arms of the vaccinia thymidine kinase (TK) gene to facilitate homologous recombination with the vaccinia virus genome.

Using tthe general method described by Mackett, M. et al. (1984) J. Virol. 49:857-8864, the pGS62/E6 and pGS62/E7 were separately inserted into the vaccinia vivirus genome within the thymidine kinase gene by homologous recombination. . The parental virus, v-NY, derived from the Wyeth smallpox vaccine (New YYork City Board of Health strain) was propagated in BSC40 cells after three plaqque purifications. Briefly, the chimeric plasmid was introduced into cells previviously infected by the parental type vaccinia virus. The TK region of the plalasmid is homologous to the TK region of the virus. The inserted plasmid recombbined, inserting the foreign gene into the vaccinia virus genome, rendering the rerecombinant virus $TK^-$. The $TK^-$ virus was selected in $TK^-$ cells grown in the presence of medium containing 5bromodeoxyuridine. The

recombinant t viruses were purified by three rounds of plaque purification and chimeric viruruses were identified by hybridization of the viral DNAs with either $^{32}$P-Labelled d E6 or E7 DNA purified from a bacterial vector.

## EXAMPLE 3

### Construction of Mammalian Expression Plasmids pCDM8/E6 and pCDM8/E7

The HHPV 16 open reading frames (ORF) for E6 and E7 were separately cloned into tlthe mammalian expression vector pCDM8 (Invitrogen, San Diego, CA) at theie Hind III site downstream of the immediate early (IE) cytomegalovivirus (CMV) promoter (FIGURES 1 and 2). The E6 ORF was subcloned byy gel-purifying the BamHI, EcoRI fragment from the pGS62/E6 vaccinia virusis expression plasmid and ligating it into the BamH1, EcoRI cleaved pIC 20H plaasmid in order to obtain a Hind III site at the 5' end of the E6 ORF for directionaal cloning into pCDM8. Plasmid pIC 20 H/E6 was digested with Hind III andd XhoI and the E6 ORF, along with untranslated sequences of of 58bp that arete 5' and 98bp that are 3' of the ORF, was gel-purified and ligated into the Hindid III, XhoI-digested pCDM8 vector. The recombinant pCDM8/E6 shown in FIGGURE 1 was isolated, characterized and propogated, as described herein above.e. The colonies formed were screened by standard miniprep DNA purification ı methods, followed by treatment of the DNAs with restriction endonucleasetes. The combinations of enzymes were selected to yield diagnostic banding patteterns of the DNA fragments cloned in the correct orientation with respect to theie direction of transcription, i.e 5' to 3'. Appropriate clones were amplified annd their DNA purified by CsCl, ethidium bromide equilibrium centrifugationn.

The E7.7 ORF was gel purified from pIC 20R/E7 as an EcoRI, PstI fragment and sisubcloned into pIC 20H at the EcoRI and PstI sites (FIGURE 2). The E7 ORF wwas removed from pIC 20H/E7 using Hind III and PstI in order to introduce a Hirind III site at the 5' end of the E7 ORF. The E7 ORF-containing fragment was i gel-purified and ligated into the Hind III, PstI-cleaved pCDM8 expression plasismid, along with 56bp untranslated HPV sequence 5' of the ORF and 24bp untraanslated HPV sequence 3' of the E7 ORF, to produce pCDM8/E7 shown in FIGUURE 2. Colonies were screened, amplified and the DNA purified as outlined abovve.

## EXAMPLE 4

### Inisertion of a DNA Encoding an Epitopic Region of an HPV Nucleoprotein into Epithelial or Fibroblast Cells

A DNAA nucleotide sequence corresponding to at least one epitopic region of either:r the E6 or E7 nucleoprotein of human papilloma virus is inserted into a mammalilian expression vector and transfected into epithelial or fibroblast cells by the mmethods described in Example 3, hereinabove, using standard calcium phosphhate precipitation techniques followed by glycerol shock. The cells are then pplaced in G418-containing Iscove's Modified Dulbecco's medium (IMDM) (1 mgg G418/ml) after transfection. When colonies grow to a visible size, they are reremoved by using cloning rings, transferred to individual wells of 24-well plates aand grown in tissue culture to higher numbers. Part was stored in liquid nitrogigen in 10% DMSO, 90% fetal calf serum and used for further studies.

## EXAMPLE 5

Radioimmunoprecipitation of E6 and E7 Gene Product from Recombinant Vaccinia Virus Infection

Rabbitit antiserum against HPV16 E7, and HPV16 E6 TrpE fusion proteins were s prepared (α16E7NP and α16E6DS, respectfully) and provided by D. Galloway s as described in Jenison et al. (1988) J. Virol. 62:2115-2123.

Rabbitit antiserum against HPV16 E6 was provided by D. Lowy (National Cancer Instituute, Laboratory of Cellular Oncogloy, Bethesda, MA) as described in Androphy e et al. 1987, EMBO 6:989.

BSC40 monkey cells infected for 12 hours with a vaccinia recombinant virus of the ppresent invention or CaSki cervical carcinoma cells (obtained from the ATCC) ı were labelled for 60 minutes in a 10 cm culture dish in methionine-freee medium supplemented with 5% dializyed fetal calf serum (FCS), 0.25 mmCi of [$^{35}$S]-methionine and 0.25 mCi of [$^{35}$S]-cysteine. The cells were lysed in ı 1 ml of lysing buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5% Nonidetet P-40, 0.5% deoxycholate, 0.5% sodium dodecylsulfate (SDS), 0.1 trypsin irinhibitor unit/ml of aprotinin, and 1 mM EDTA) and briefly sonicated.

The lysysate was pre-cleared by incubation at 4°C for 1 hour with 10 μl of normal rabbit t serum or vaccinia immune rabbit serum and protein A-Sepharose beads. After c centrifugation the beads were discarded. The cleared lysates were incubated withh rabbit αE6 (D. Lowy) or E7 (α16E7NP) immune serum that had been preadsorbed by incubation with unlabelled vaccinia virus lysate. Protein A-coated Sephharose beads were then added to the mixture of immune rabbit antibody and ı cell lysate, and incubated. After centrifugation the beads were washed twice ı with RIPA buffer (10 mM Tris-HCl(pH 7.4), 0.15 M NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS, 0.1 trypsin inhibitor unit/ml of aprotinin)

and then consecutively with high-salt buffer (10 mM Tris-HCl(pH 7.4), 2 M NaCl, 1% NP-40, 0.5% deoxycholate), low salt buffer (0.5% NP-40, 0.1% SDS in PBS), , 1 M $MgCl_2$, 1 M Tris-HCl(pH 7.4) and RIPA buffer.

The proteins were released from the antibodies and beads by boiling for 5 minutes in sample buffer and analyzed by 17.5% SDS-PAGE in comparision to prestained standard molecular weight markers.

Autoradiography of the gels demonstrated the presence of a band of about 17 kDa a molecular weight in the E6-vaccinia recombinant lysates when anti-E6 rabbit serum (provided by D. Lowy) was used to precipitate the lysates. (FIGURES 3 a and 4). No band was seen in the corresponding position in either lanes when the precipitation was performed with normal rabbit serum or the lanes where vNNY lysates were precipitated with anti-E6 rabbit serum.

Two vaccinia E6 recombinant plaques, 7.1 and 8.1 were expanded and analyzed with s similar results.

When the E7-vaccinia recombinant lysates were analyzed by autoradiography, as described above, a band of 18-20 kDa was found upon precipitation with anti-E7 rabbit serum ($\alpha$16 E7 NP). No band was seen in the corresponding position in either of the lanes where the precipitation was performed with normal rabbit serum or the lanes where radiolabelled vNY lysates were precipitated with anti-E7 rabbit serum. A pulse-chase study (FIGURE 5) demonstrated that E7 protein is degraded 2-6 hours after synthesis. Similar results have been shown for the E7 protein in CaSki cells (D. Smotkin and F. Wettstein, (1987) J. Virol. 61:1686-1689) that carry the entire HPV16 genome. The migration of the E7 proteins appears identical to that seen in the CaSki lysate, suggesting that a full length gene product is made in the recombinant vaccinia cells.

## EXAMPLE 6

### Radioimmunoprecipitation of the E7 Gene Product from Recombinant COS Cells

COS monkey cells were transfected with the pCDM8/E7 plasmid of the present invention and grown in culture for 48 hours. The cells were then labelled with $^{35}$S-Met and $^{35}$S-Cys as described in EXAMPLE 5 for CaSki cells. The cells were then partitioned into nuclear, cytosol and membrane fractions as described in Sato et al. (1989) Virology 170:311-315.

The proteins were released by boiling for 5 minutes in sample buffer, as described in EXAMPLE 5, and analyzed by 17.5% SDS polyacrylamide gels in comparision to prestained standard molecular weight markers. Autoradiography of the gels demonstrated the presence of a band at about 18 kDa upon precipitation with anti-E7 rabbit serum. The results are shown in FIGURE 6.

## EXAMPLE 7

### Western Blot Analysis

The Western blot study of the immunogenicity of the E6 and E7 peptides was performed using fusion proteins composed of TrpE linked to segments of the HPV16 E6 or E7 ORFs as described in Jenison et al. (1988) J. Virol. 62:2115-21233.

Briefly, fusion proteins were partially purified by means of their relative insolubility in nonionic detergents. A 50 ml induced bacterial culture was pelleted, suspended in 10 ml of Tris-EDTA buffer (50 mM Tris (pH 8.0)-5 mM EDTA) and digested at 0-4° C for 90 minutes with lysozyme (2 mg/ml).

NaCl (5.0 M) and 10% NP-40 were added to a final concentration of 0.3 M and 0.7%, respectively, and the mixture was maintained at 0-4°C for 30

minutes. The : solution was passed through an 18-gauge needle three times to reduce its viscoosity, and was maintained at 0-4°C for additional 30 minutes.

The insoluble fraction was pelleted at 16,000 x g at 4°C for 10 minutes, resuspended in ı 10 ml of 10 mM Tris(pH 8.0)-1.0 M NaCl, and maintained at O to 4°C for 10 minutes. The insoluble fraction was pelleted, as described above, resuspended inn 1.0 ml of Laemmli protein sample buffer (Laemmli (1970) Nature 227:6800) (10 ml 0.625 M TRIS pH 6.8, 20 ml 10% SDS, 20 ml glycerol, 2 ml l 2-mercaptoethanol, 1 ml 1.5% Bromophenol Blue (prepared in 70% reagent alcohol), 1 ml 1.0% Pyronin Y (Biorad Catalog No. 161-0425, or equivalent, prepared in $H_20$), and 36 ml deionized $H_20$) and heated to 100°C for 5 minutes.

Fusion protein preparations were separated by electrophoresis through 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS). The fusion protein was quantitated by visual inspection of Coomassie blue-stained gels. The volume of each quantity of insoluble protein fraction loaded per 6 mm slot was adjusted to give a Coomassie blue-staining intensity equivalent to 5 $\mu$g of bovine serum albumin. Proteins were transferred to nitrocellulose in 25 mM Tris-195 mM glycine (pH 8.5)-20% methanol (Western transfer buffer) at 100 mA for 16-18 hours. Blots were soaked for 10 minutes in phosphate-buffered saline containing 10 mM N-ethylmaleimide, incubated for 2 hours in 5% nonfat dry milk, 0.9% NaCl, 0.1% Antifoam-A (Sigma Chemical Co., St. Louis, , Mo.), 0.1% sodium azide and 1 mM potassium iodide (blotto) and then incubated for 1 hour in blotto containing 10% fetal calf serum.

Blocking reagent was prepared from a 50 ml induced bacterial culture which was pelleted, resuspended in 3.6 ml of 50 mM Tris (pH 8.5), 5 mM EDTA, and sonicated at 50 W for 20 seconds; 0.4 ml of 20% SDS was added, and the lysate was heated to 100 ° C for 5 minutes. This reagent termed

"Blocko" was stored at (-20°C) in aliquots. To make the TrpE protein sample run as a negative control antigen for Western blots, the lysed induced bacterial culture containing the vector (path 10) only was pelleted as described to make "Blocko". This cell lysate was mixed 1:1 with 2X Laemmli sample buffer, boiled 5 minutes and run on gels to view the Trp control protein.

To make blocking reagent, this mixture ("Blocko") was diluted 1:20 in blotto, and NP-40, and sodium deoxycholate were added to a final concentration of 0.1% each. Rabbit sera were diluted 1:100 in 2.5 ml blocking reagent and preincubated at 4°C for 8 hours. Nitrocellulose blots were then added, and incubation at 4°C was continued for 16-18 hours. The blots were washed three times in 0.5% deoxycholate, 0.1 M NaCl, 0.5% Triton X-100, and 10 mM sodium phosphate (pH 7.5) for 20 minutes per each wash. Goat anti-rabbit serum conjugated with alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, IN) was added diluted 1:1000 in blotto. After a 2 hour incubation at about 27°C, the filters were again washed three times and transferred to the solution of substrate (TABLE 1) for 10 minutes or until color developed. The reaction was stopped by rinsing the filters in distilled water. The filters were dried and photographed.

## TABLE 1

## WESTERN BLOT REAGENTS

| 10X TRANSFER BUFFER | 2 LITERS |
|---|---|
| Trizma base (25 mM Tris) | 60.53 g |
| Glycine (1.95 mM) | 288.27 g |

Upon dilution to 1X, add MeOH to 20% of final volume.

| BLOTTO | 4 LITERS | 8 LITERS |
|---|---|---|
| Nonfat powdered milk (5%) | 200 g | 400 g |
| NaCl (0.9%) | 36 g | 72 g |
| Antifoam A (0.1%) | 12 ml | 24 ml |
| Sodium azidide (0.1%) | 4 g | 8 g |
| Potassium iodiodide (1 mM) | 0.664 g | 1.33 g |

| WESTERN WASH | 4 LITERS | 8 LITERS |
|---|---|---|
| Deoxycholic acid (0.5%) | 20 g | 40 g |
| Triton X-100 (0.5%) | 20 ml | 40 ml |
| NaCl (0.1 M) | 23.4 g | 46.8 g |
| 1 M Sodium phosphate, pH 7.4 (10 mM) | 40 ml | 80 ml |

(1 M sodium phosphate - 268 g $Na_2HPO_4 7H_2O$ [heat to dissolve], approx. 4 ml 85% $H_3PO_4$ to pH 7.4, q.s. to 1 liter)

ALKALINE PHOSPHATASE SUBSTRATE

Enough for 2-2 blots. Mix after addition of each reagent. Protect from light; use within 1 hour.

| | |
|---|---|
| Alkaline phosphatase buffer | 10 ml |
| NBT substrate (50 mg/ml in 70% dimethylformamide) | 66 µl |
| BCIP substrate (50 mg/ml in 100% dimethylformamide) | 33 µl |

ALKALINE PHOSPHATASE BUFFER

| | | |
|---|---|---|
| 1 M Tris, pH 9.5 | 10 ml | (100 mM Tris-HCl, pH 9.5) |
| 5 M NaCl | 2 ml | (100 mM NaCl) |
| 1 M $MgCl_2$ | 0.5 ml | ( 5 mM $MgCl_2$) |

Q.S. to 100 ml

NBT SUBSTRATE Nitro Blue Tetrazolium (Sigma)

BCIP SUBSTRATE 5 Bromo-4-chloro-3-indoyl Phosphate (Sigma)

## EXAMPLE 8

HPV Peptides

Synthetic peptides corresponding to specific regions of either the E6 or E7 nucleoprotein of HPV16 were synthesized. The specific peptides are listed in Table 2, and correspond to the designated amino acid residues, read from the amino terminus to the carboxy terminus, in the sequence of the E6 or E7 proteins, as illustrated in FIGURE 7.

TABLE 2

| Peptide | E6 or E7 | Amino Acid Position |
|---|---|---|
| 359 | E7 | 29 - 50 |
| 360 | E7 | 70 - 81 |
| 361 | E7 | 1-10 |
| 357 | E6 | 148-158 |
| 358 | E6 | 119- 134 |
| 375 | E6 | 8-20 |
| 376 | E6 | 1- 20 |

## EXAMPLE 9

Monoclonal Antibody Production
Using HPV16 Peptides as Immunogen

Twenty micrograms of peptide 359 conjugated to keyhole limpet hemocyanin (KLH) was emulsified in complete Freund's adjuvant and administered to mice subcutaneously and intraperitoneally. Approximately 3 and 5 1/2 weeks later, booster injections were given intraperitoneally in incomplete Freund's adjuvant. Spleen cells were harvested after 3 days and fused with the AG8 myeloma line by standard hybridoma techniques, (see Milstein, supra.). Supernates from healthy clones were screened for the

presence of specific antibody in ELISA assays using plates coated with the unconjugated peptitide at 500 nanograms per well in 0.1 M carbonate buffer, pH 9.6. Goat anti-mouse IgG conjugated to horse radish peroxidase (HRP) was added after 3 washes, and substrate reactions were performed by standard ELISA methodology. Two highly reactive clones (clones #10 and #14) were chosen for further cloning and their supernatants were titrated in the peptide 359 ELISA with the result seen in FIGURE 8. Similarly, monoclonal antibodies have been prepared against E6 peptides 358 and 375 (data not shown).

## EXAMPLE 10

Peptide - KLH Immunogenicity Studies

Rabbits were immunized by intradermal administration of 100 $\mu$g of the peptide conjugated to KLH, mixed with complete Freund's adjuvant. The animals were boosted three weeks later by intradermal administration of 50 $\mu$g of the peptide conjugated to KLH, mixed with incomplete Freund's adjuvant. The rabbits were bled three days after this boost.

Sera was stored at (-20°C). Rabbit antisera against Trp E-HPV fusion proteins were prepared as described in Jenison et al. (1988) J. of Virol. 62:2115-2123, and were provided by Dr. Denise Galloway (Fred Hutchinson Cancer Research Center, Seattle, WA). To eliminate antibodies directed against E. coli-encoded proteins (including the Trp E portion of the fusion proteins) sera were preabsorbed against denatured lysates of E. coli which express vector (pATH) sequences, using the Blocking reagent described in EXAMPLE 7 hereinabove.

Rabbit sera were serially diluted and reacted in Western blot assays against Trp E-E6 and E7 fusion proteins. All anti-peptide sera were reactive

with the homnologous fusion proteins, demonstrating the immunogenicity of peptide conjugigates. The titer of antisera is expressed herein as the reciprocal of the highest dililution showing reactivity with the specific protein in Western blot analysis. Thene titers of the various antisera are shown in Table 3 and the Western blots e of the titrations are seen in FIGURES 9 and 10.

TABLE 3

| Rabbit S Sera | Titer* | | |
|---|---|---|---|
| α357 (EE6) | α3,200 | 100 | ** |
| α358 (EE6) | 102,400 | α6,400 | |
| α359 (EE7) | α409,600 | α409,000 | |
| α360 (EE7) | 1,600 | | |
| α361 (EE7) | 800 | | |
| α16E7NNP | 720,000 | | |
| α16E6DDS | 40,000 - 80,000 | | |

* Highest it reciprocal dilution showing positive staining of E6 or E7 band.
** Two titeters indicates two different rabbits were tested.

The annti-peptide 359 antiserum was the most reactive of the antisera tested in thesee Western blots, having a titer of α409,600. The specificity of these reactions s were demonstrated by reacting two serum dilutions with both the homologous TrTrp fusion protein and with the Trp control antigens in Western blots. The serera were found to be specific for the homologous fusion protein (FIGURE 11).. Titers for the anti-peptide 359 antisera were α1,000,000 in this assay.

Positivee control rabbit sera (α16E7NP and α16E6DS) were prepared against Trp E-3-E6 fusion protein, pl6 E6 DS-2 (DraIII(111)-Sau3a(525)) and

against E7 fusiion protein, pl6 E7 NPI (Nsi I (562)-PstI(875)) respectively, and provided by D.). Galloway, Fred Hutchinson Cancer Research Center, Seattle, WA.

## EXAMPLE 11

### Identification of B Cell Epitopes bv ELISA

KLH conjugated E6 and E7 peptides, as described in Example 10, were used in an ELISSA assay to determine whether they represent antigenic epitopes, recognized by animals immunized with bacterially expressed HPV16 E6 or HPV16 E7 fusiion proteins.

The threee E7 peptides, described in EXAMPLE 8, were recognized by rabbit antisera against Trp E-E7 (α16 E7 NP) (provided by D. Galloway, Fred Hutchinson Cancer Research Center, Seattle, WA), as seen in Table 4. A monoclonal antibody recognizing HPV16 E7 purchased from Triton Biosciences was mapped to the aminoterminal peptide 361.

TABLE 4

Titer* of antiserum tested in EUSA against KLH-conjugated peptides from the E7 open reading frame.

| Peptide | α16 E7 NP | mcAb Triton Bioscience |
|---|---|---|
| 359(aa 299-50) | 1,600 | < 100 |
| 360(aa 700-81) | 6,400 | < 100 |
| 361(aa 1-:.-10) | 12,800 | α12,800 |

* Titer was detetermined as the reciprocal of the highest serum dilution showing four-fold higigher ELISA values than background. Background was determined uusing normal rabbit serum at 1:100 on the same peptide-coated plates.

Two peptides from the E6 sequence (357 and 358) were similarly studied. These results are shown in Table 5 for use of two different anti-E6 antisera. Peptide number 358 is reactive with HPV16-E6 (Lowy) at a serum dilution of 1:400, while α16E6DS has a lower reactivity (1:100). Peptide 357 was weakly recognized by α16E6DS at a 1:100 serum dilution.

TABLE 5

Titers* of antisera tested in ELISA against KLH-conjugated peptides of the E6 open reading frame.

| Peptide | α16 E6 DS | αHPV16-E6 (Lowy) |
|---|---|---|
| $357_{(aa\,148\text{-}158)}$ | 100 | <100 |
| $358_{(aa\,119\text{-}134)}$ | 100 | 400 |

* Titers were determined as the reciprocal of the highest dilution showing EUSA values three times background values, with background determined as in Table 4.1.

## EXAMPLE 12

### Recognition of Native E7 Protein By Anti-Peptide Antisera

Rabbits were immunized with E7 peptides, as described in Example 8, according to the method of Example 10 and as assayed by RIP. The results shown in FIGGURE 12 illustrate the recognition of the E7 peptide by anti-E7 peptide antisera. Antisera from rabbits immunized with peptide 359, corresponding to amino acid residue 29 to 50 of E7, recognized the native E7 protein in vaccinia recombinant lysates as determined by radioimmunoprecipitation.

NOT TO BE CCONSIDERED FOR THE PURPOSE OF PUBLICATION

## EXAMPLE 13

Reverse Transcriptase-Polymerase Chain Reaction Analysis of RNA

Twenty-four clones each of the M2 murine melanoma cells and NCTC 2555 fibroblast cells that were transfected with the pCDM8/E7 plasmid as described in Example 3, hereinabove, were examined by RNA dot blot assay. Three of these clones which gave positive signals were then further examined by reverse transcriptasepolymerase chain reaction (RT-PCR) analysis.

Cytoplasmic RNA from these transfected cells were isolated as described in Ausubel et al. (1989) in Current Protocol in Molecular Biology, Greene Publishing Associates.

Typical primer extension-RT of cytoplasmic RNA to synthesize first strand cDNA was used, together with PCR to amplify the cDNA. One $\mu$g of the cytoplasmic RNA was used as a template for the amplification reactions. The first strand cDNA was synthesized by using murine leukemia virus reverse transcriptase (Life Sciences).

RT buffer containing denatured RNA samples, 1 $\mu$g denatured random hexamer, 1 mM of each deoxynucleotide triphosphate (dNTP), 10 mM sodium pyrophosphate, 5 mM dithiothreitol, 10 units of RNasin (Promega, Madison, WI) and 18 units of murine leukemia reverse transcriptase was maintained for one hour at 42°C, and subsequently denatured at 100°C for 10 minutes. The supernatant was used for PCR.

The oligonucleotide primers used for PCR were HPVA22:5'-GCATGGAGATACACCTACATTG-3' and HPVA20: 5' -TGGTTTCTTGAGAACAGATGG-3' (DNA Factory, San Diego, CA). The cDNA fragments of 292 bp were amplified. The PCR reaction mixture (GeneAmp DNA amplification Reagent Kit, Perkin Elmer Cetus, Norwalk, CT)

contains 200 μM dNTP, 1 μM primer HPV A22 and HPV A20, various cDNA synthesized by RT and 2.5 units of Taq polymerase. One ng of pCDM8/E7 plasmid was used in the PCR as a positive control. PCR (denaturation at 94°C for 1 minute, annealing at 50°C for 2 minutes and extension at 72°C for 3 minutes) was performed by a DNA Thermal Cycler (Perkin Elmer Cetus) in 33 cycles and 20 μl of PCR products were fractionated by electrophoresis on a 4% NuSieve agarose gel (FMC Bioproducts, Rockland, ME) stained with ethidium bromide.

The results shown in FIGURE 13 illustrate that the PCR products from three transfectants (E7C3, N7.2 and N7.4) display the predicted 292 bp DNA fragments. The parental cell lines (melanoma and NCTC 2555 fibroblasts cells), however, do not display these fragments.

When the mRNA isolated from these transfectants was pretreated with DNase-free RNNase before RT-PCR, the PCR product did not display these fragments, as monitored by gel electrophoresis.

## EXAMPLE 14

### Tumor Induction and Regression

The ability of immunization with HPV transfected cells to protect against tumor development was studied in female C3H/HeN mice, 6-10 weeks old (Charles River Breeding Laboratories).

Groups of 5 mice each were injected intraperitoneally with a non-tumorigenic transfectant, derived from NCTC 2555 fibroblast cells, expressing either an HPV-E6 or -E7 epitope and expressing a high level of major-histocompatibility complex (MHC) class I antigen. The mice were then challenged by subcutaneous administration of a tumorigenic dose ($4 \times 10^6$ cells) of M2 melanoma cells transfected with HPV16 E7 (E7C3) on the shaved right

Injection of C3H/HeN mice with E7C3 cells produced tumors in the mice. When interferon was concurrently administered to these mice, quick regression of the tumors occurred; however, interferon administered to mice injected with the non-transfected M2 cells did not inhibit tumor development.

Studies in nude mice showed that functional T cells are needed for tumor regression to occur. Tumors developed in nude mice in response to inoculation with either transfected or non-transfected M2 cells. The results of this study are shown in Table 7. Further, the presence or absence of interferon had no effect upon tumor development in nude mice.

TABLE 7

| INOCULUM | MICE | PROGRESSIVE TUMOR GROWTH |
|---|---|---|
| M2 | C3H/HeN | Yes |
| M2 + Interferon | C3H/HeN | Yes |
| E7C3 | C3H/HeN | Yes |
| E7C3 + Interferon | C3H/HeN | No |
| M2 | Nude | Yes |
| E7C3 | Nude | Yes |
| E7C3 + Interferon | Nude | Yes |
| M2 + Interferon | Nude | Yes |

## EXAMPLE 15

### CD8 + T Lymphocyte Mediation of Tumor Regression

C3H/HeN mice were immunized with NCTC 2555 derived non-tumorigenic fibroblasts transfected with HPV16 E7 (N7.2). These mice were then each injected intraperitoneally with 1.0 ml of PBS-diluted ascites fluid containing 1 mg of an anti-CD8 monoclonal antibody (clone 116-13.1 IgG2A from ATCC) to deplete the $CD8^+$ T lymphocytes. Control mice

We Claim:

1. A composition comprising an immunogenic peptide that substantially corresponds to the amino acid residue sequence of a peptide expressed in a mammalian cell in response to human papilloma virus infection.

2. The composition of Claim 1, comprising a recombinant cell containing a gene encoding said peptide.

3. The composition of Claim 2, wherein said recombinant cell is a virus.

4. The composition of Claim 3, wherein said virus is a vaccinia virus.

5. The composition of Claim 2, wherein said recombinant cell is selected from the group consisting of epithelial cells, fibroblasts and MHC class I positive lymphocytes.

6. The composition of Claim 2, wherein said recombinant cell is a tumor cell.

7. The composition of Claim 2, wherein said gene encodes a peptide substantially corresponding to a region of the E6 nucleoprotein.

8. The composition of Claim 2, wherein said gene encodes a peptide substantially corresponding to a region of the E7 nucleoprotein.

9. The composition of Claim 1, wherein said peptide comprises an epitopic region of the E6 protein.

10. The composition of Claim 1, wherein said peptide comprises an epitopic region of the E7 protein.

11. The composition of Claim 4, comprising a recombinant vaccinia virus containing a gene that encodes and expresses an epitopic region of an E6 nucleoprotein of human papilloma virus.

12. The composition of Claim 4, comprising a recombinant vaccinia virus containing a gene that encodes and expresses an epitopic region of an E7 nucleoprotein of human papilloma virus.

13. The composition of Claim 4 comprising a recombinant vaccinia virus containing a region of a gene of human papilloma viruses that induces the expression of an immunogenic peptide in a mammalian cell.

14. The composition of Claim 6, wherein said tumor cell comprises a recombinant mammalian cell containing a region of the E6 gene of human papilloma virus that encodes and expresses an epitopic region of an immunogenic peptide by said mammalian cell.

15. The composition of Claim 14, wherein said mammalian cell is a human cell.

16. The composition of Claim 6, wherein said mammalian cell comprises a recombinant human cell containing a region of the E7 gene of human papilloma virus that encodes and expresses an epitopic region of an immunogenic peptide by said melanoma cell.

17. The composition of Claim 16, wherein said mammalian cell is a cell selected from the group consisting blood cells, fibroblasts, and epithelial cells.

18. A method of treating a condition resultant from human papilloma virus infection comprising:

a administering to a patient a therapeutically effective amount of a c composition, comprising a peptide that substantially corresponds t to the amino acid residue sequence of a region of a peptide e expressed in mammalian cells in response to a human papilloma v virus infection, for a time period sufficient to inhibit the p progression of said condition.

19. The method of Claim 18, wherein said composition comprises a recombinant cell containing a region of an E6 gene of human papilloma virus that induces the expression of an immunogenic peptide in a mammalian cell.

20. The method of Claim 19, wherein said cell is a vaccinia virus.

21. The method of Claim 18, wherein said composition comprises a recombinant cell containing a region of an E7 gene of human papilloma

virus t that induces the expression of an immunogenic peptide in a mammmalian cell.

22. The meethod of Claim 21, wherein said cell is a vaccinia virus.

23. The meethod of Claim 18, wherein said condition is a cervical wart.

24. The mmethod of Claim 18, wherein said condition is a human cervical carcinooma.

25. The meethod of Claim 18, wherein said method inhibits the proliferation of cellsls infected with human papilloma virus.

26. A propiphylactic method to inhibit tumor initiation of cells following the detectiion of human papilloma virus infection comprising:

administering to a patient a therapeutically effective amount of a composition, comprising a peptide that substantially corresponds to the amino acid residue sequence of a peptide induced in a mammalian cell by human papilloma virus infection to elicit a protective response in said patient that inhibits tumor initiation of said virus infected cells.

27. The mmethod of Claim 26, wherein said composition comprises a recombbinant cell containing a gene encoding said peptide.

28. The meethod of Claim 27, wherein said recombinant cell is a vaccinia virus.

29. The method of Claim 27, wherein said recombinant cell is selected from the group consisting of epithelial cells, fibroblasts and MHC class I positive lymphocytes.

30. The method of Claim 26, wherein said peptide comprises an epitope of the E6 nucleoprotein of human papilloma virus.

31. The method of Claim 26, wherein said peptide comprises an epitope of the E7 nucleoprotein of human papilloma virus.

32. The method of Claim 26, wherein said peptide comprises a mammalian peptide induced by a region of a human papilloma virus gene.

33. A recombinant cell containing a gene encoding a peptide that substantially corresponds to an amino acid residue sequence of a peptide expressed in a mammalian cell in response to human papilloma virus infection.

34. The recombinant cell of Claim 33, wherein said peptide is expressed as an epitopic region by said cell.

35. The recombinant cell of Claim 33, wherein said cell is a vaccinia virus.

36. The recombinant cell of Claim 33, wherein said cell is an epithelial cell.

37. The recombinant cell of Claim 33, wherein said cell is a tumor cell.

38. A peptptide comprising a sequence of about 8 to about 30 amino acid residueres substantially corresponding to a region of a protein of human papillooma virus.

39. The peeptide of Claim 38, wherein said region is an epitopic region of said prrotein.

40. The peeptide of Claim 38, wherein said protein is the E7 protein of HPV166.

41. The peeptide of Claim 40, corresponding to the amino acid residue sequennce from about residue 1 to about residue 10 in the E7 amino acid sequennce of Figure 7.

42. The peeptide of Claim 40, corresponding to the amino acid residue sequencce from about residue 29 to about residue 50 in the E7 amino acid sequencce of Figure 7.

43. The peeptide of Claim 40, corresponding to the amino acid residue sequencce from about residue 70 to about residue 81 in the E7 amino acid sequencce of Figure 7.

44. The peeptide of Claim 40, wherein said protein is the E6 protein of HPV166.

45. The peptide of Claim 44, corresponding to the amino acid residue sequence from about residue 1 to about residue 20 in the E6 amino acid sequence of Figure 7.

46. The peptide of Claim 44, corresponding to the amino acid residue sequence from about residue 8 to about residue 20 in the E6 amino acid sequence of Figure 7.

47. The peptide of Claim 44, corresponding to the amino acid residue sequence from about residue 119 to about residue 134 in the E6 amino acid sequence of Figure

48. The peptide of Claim 44, corresponding to the amino acid residue sequence from about residue 148 to about residue 158 in the E6 amino acid sequence of Figure 7.

49. A method of inhibiting human papilloma virus infection in a patient comprising:

administering a sufficient amount of a immunogenic composition to a patient to effectively elicit an immunologically protective response in said patient to an infection by human papilloma virus, said immunogenic composition comprising a recombinant cell containing a gene encoding a peptide that substantially corresponds to an amino acid residue sequence of a region of a peptide induced in a mammalian cell in response to a human papilloma virus infection, said peptide or a combination thereof.

50. The method of Claim 49, wherein said recombinant cell is a virus.

51. The method of Claim 50, wherein said virus is a vaccinia virus.

52. The method of Claim 50, wherein said recombinant cell is selected from the group consisting of epithelial cells, fibroblasts and MHC class I positive lymphocytes.

53. The method of Claim 49, wherein said peptide comprises an epitopic region of an E6 nucleoprotein of human papilloma virus.

54. The method of Claim 49, wherein said peptide comprises an epitopic region of an E7 nucleoprotein of human papilloma virus.

55. An antibody molecule capable of competing with a peptide, expressed in response to an HPV infection, for a receptor for said peptide.

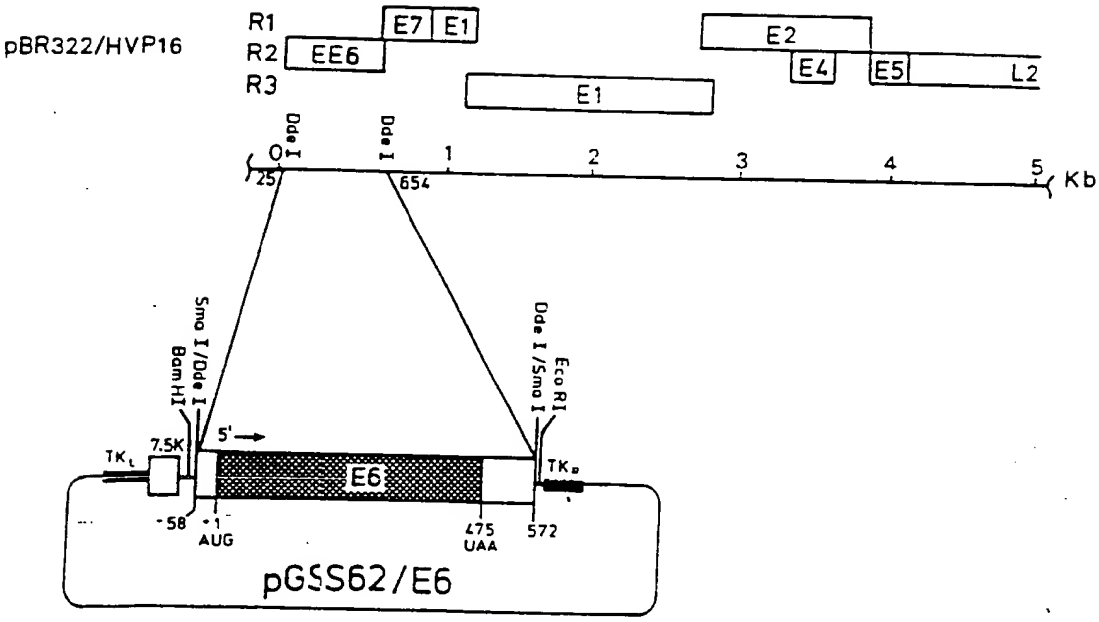
56. The antibody of Claim 55, wherein said peptide comprises a region of the E7 protein of HPV.

57. The antibody of Claim 56, wherein said antibody molecule is an anti-idiotypic antibody to said peptide.

58. The antibody of Claim 57, wherein said peptide corresponds to the amino acid residue sequence from about residue 29 to about residue 50 in the E7 amino acid sequence of Figure 7.

59. The anntibody of Claim 55, wherein said peptide comprises a region of the E6 6 protein of HPV.

60. The anntibody of Claim 59, wherein said antibody molecule is an anti-idiotypipic antibody to said peptide.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E7
E1
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0
25
654
1
2
3
4
5
Kb
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Figure 1A

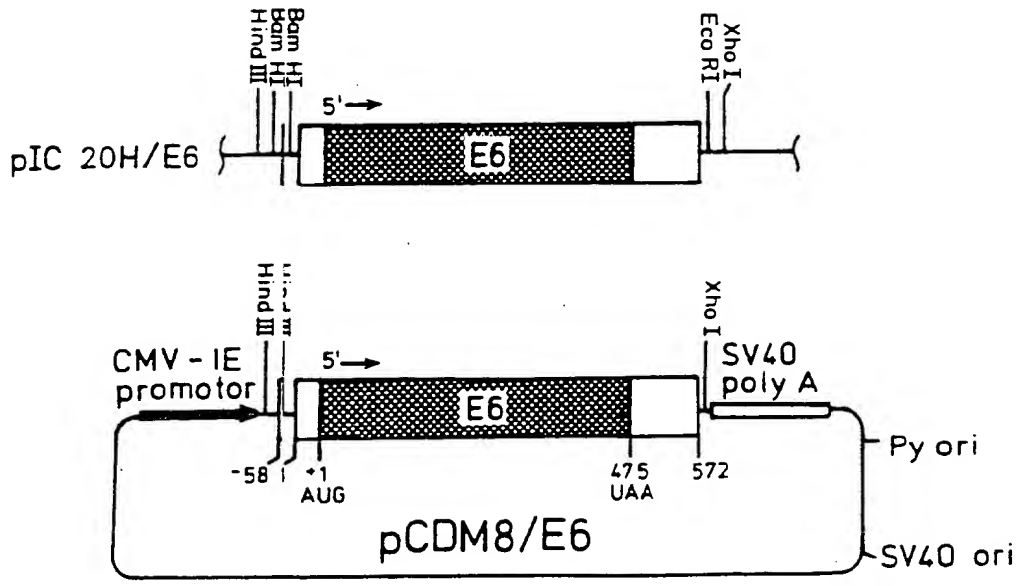
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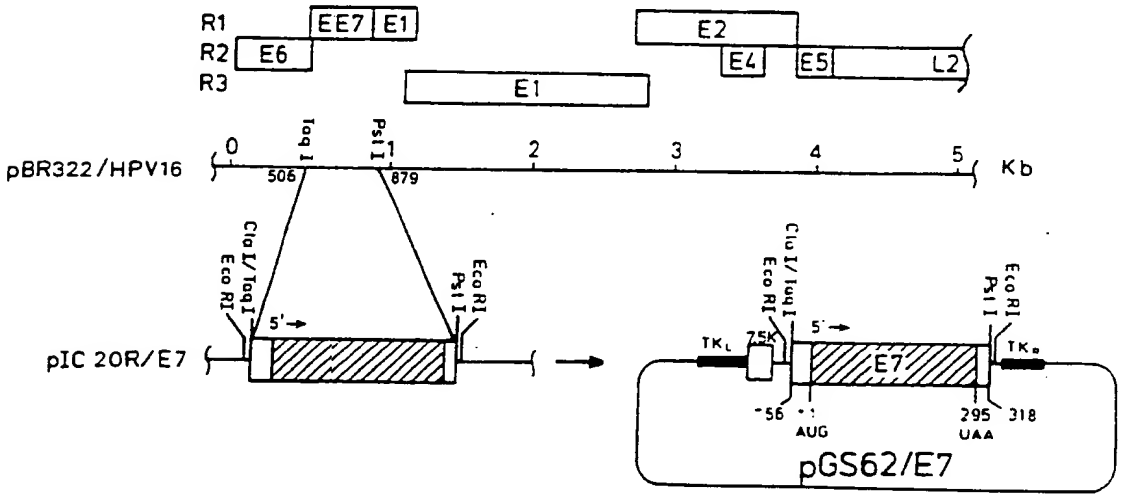
Figure 1B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UAA
295
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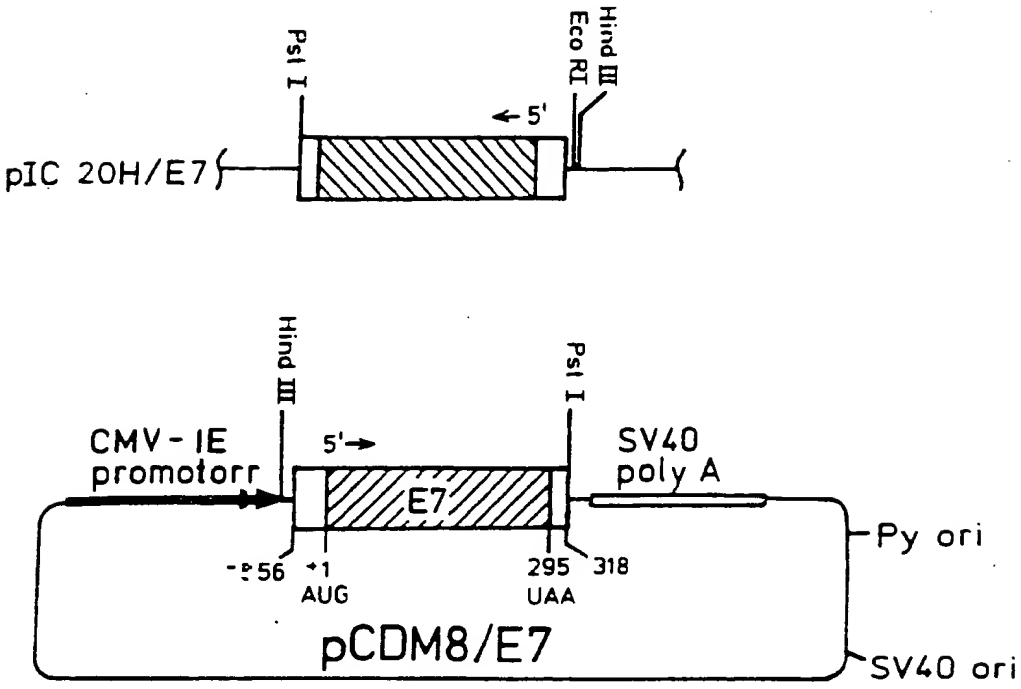
Figure 2A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pCDM8/E7
SV40 ori

Figure 2B

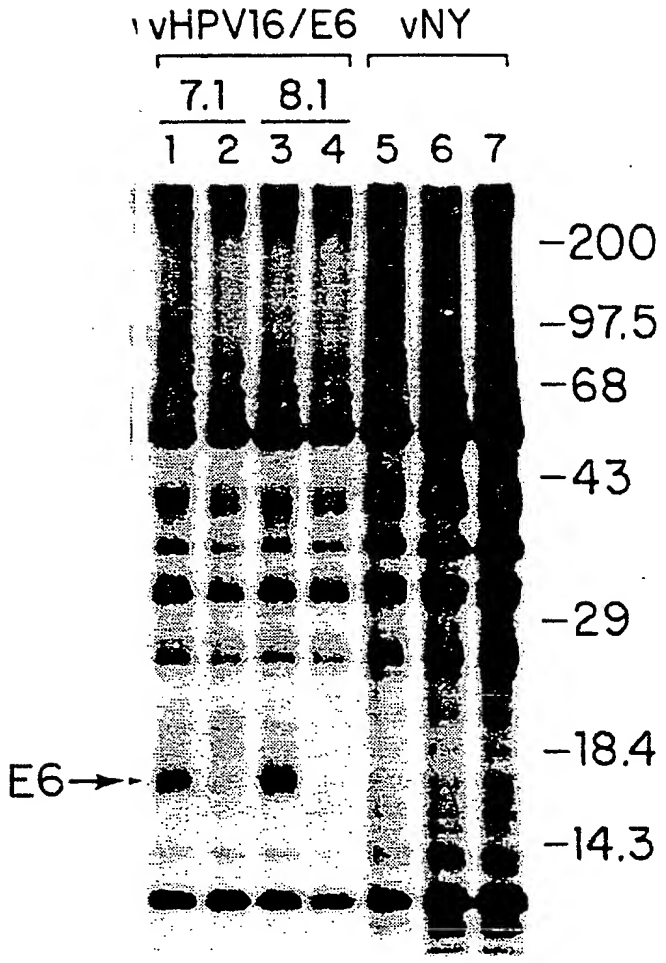



Figure 3

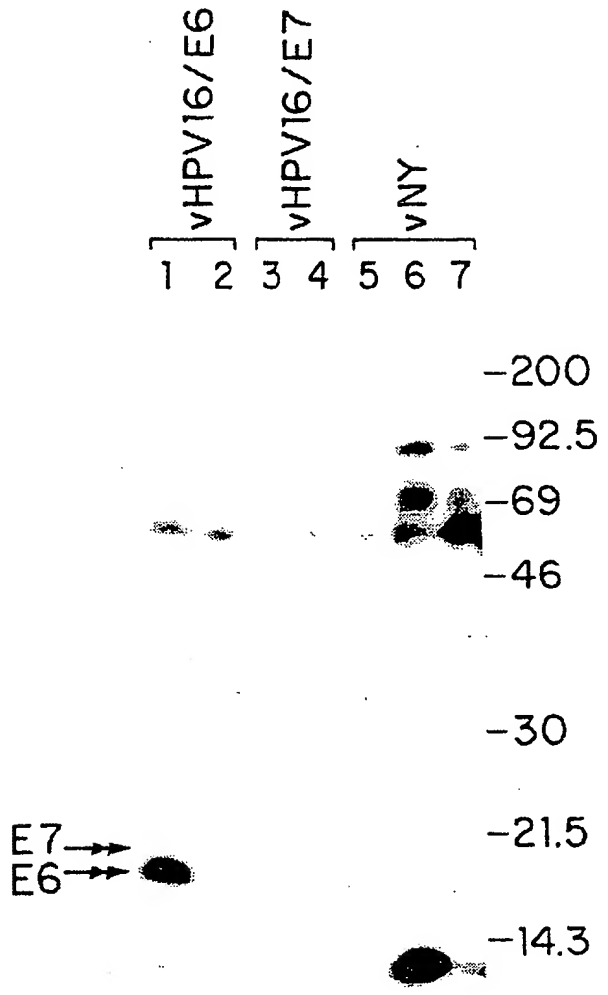



Figure 4

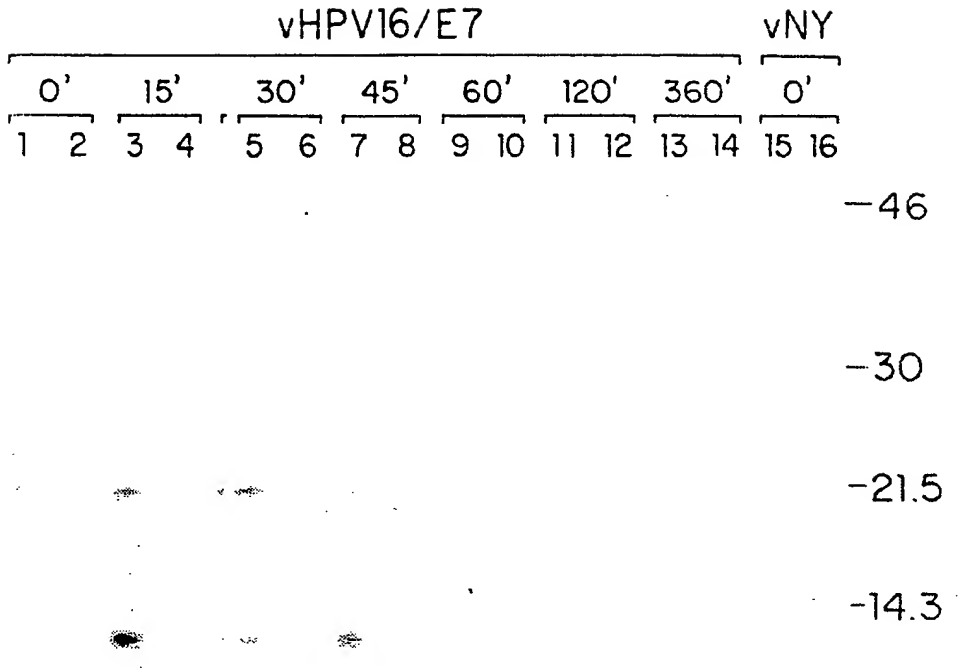

vHPV16/E7
vNY
0'
15'
30'
45'
60'
120'
360'
0'
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
-46
-30
-21.5
-14.3

Figure 5

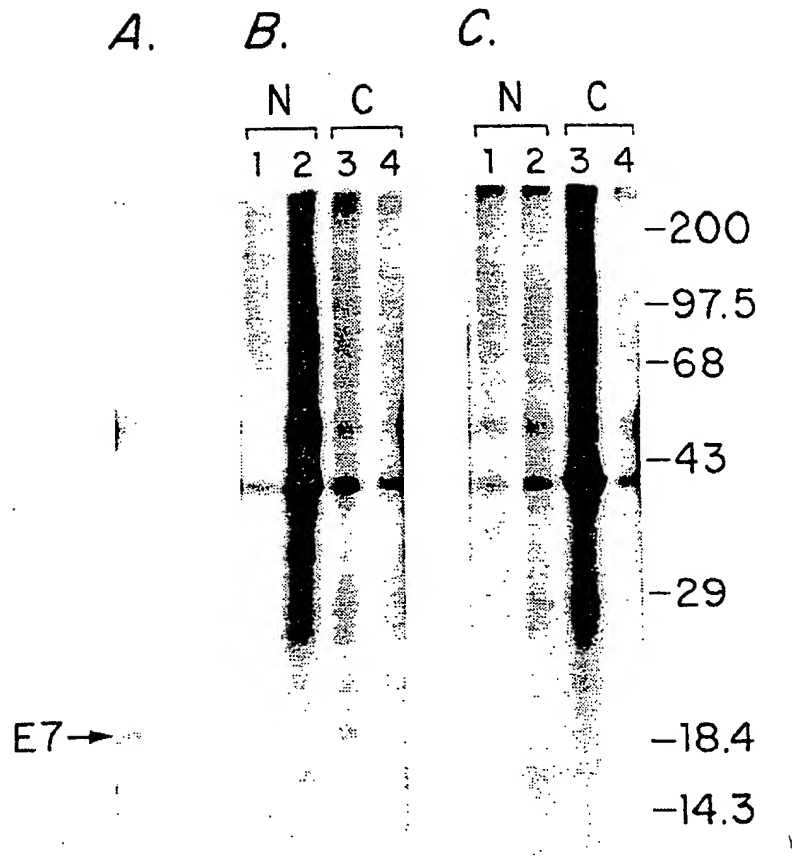
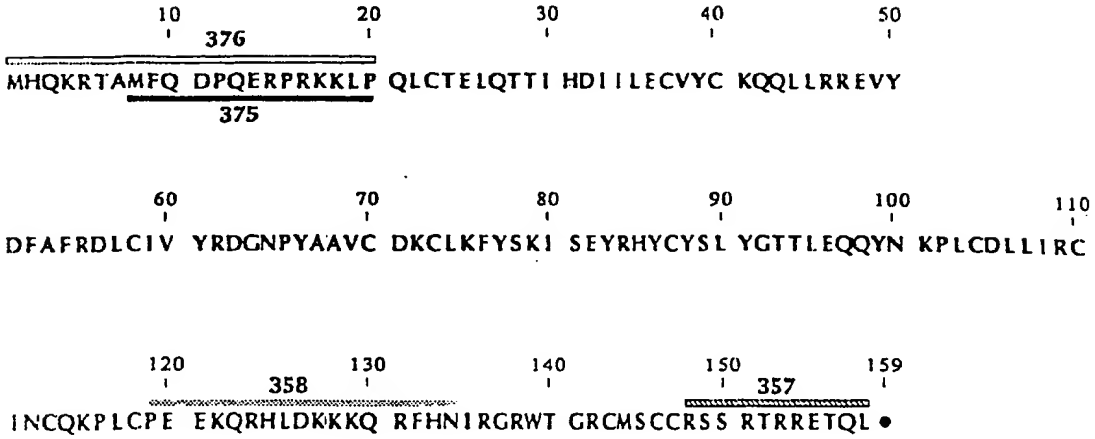



Figure 6

SUBSTITUTE SHEET

# E6 AMINO ACID SEQUENCE




# E7 AMIINO ACID SEQUENCE

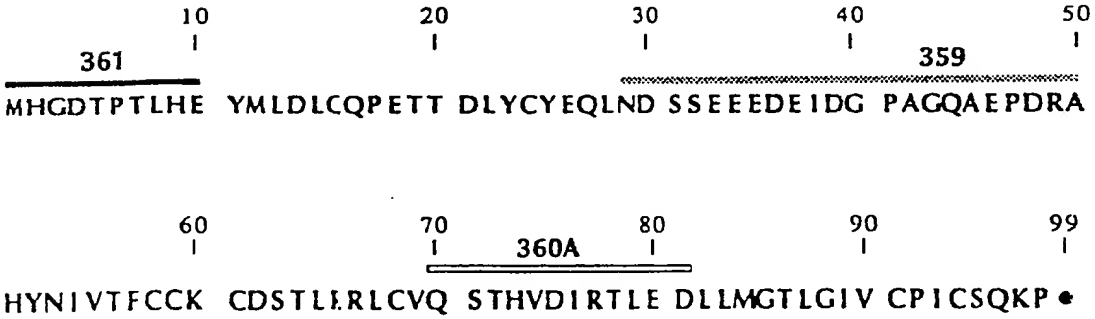



Figure 7

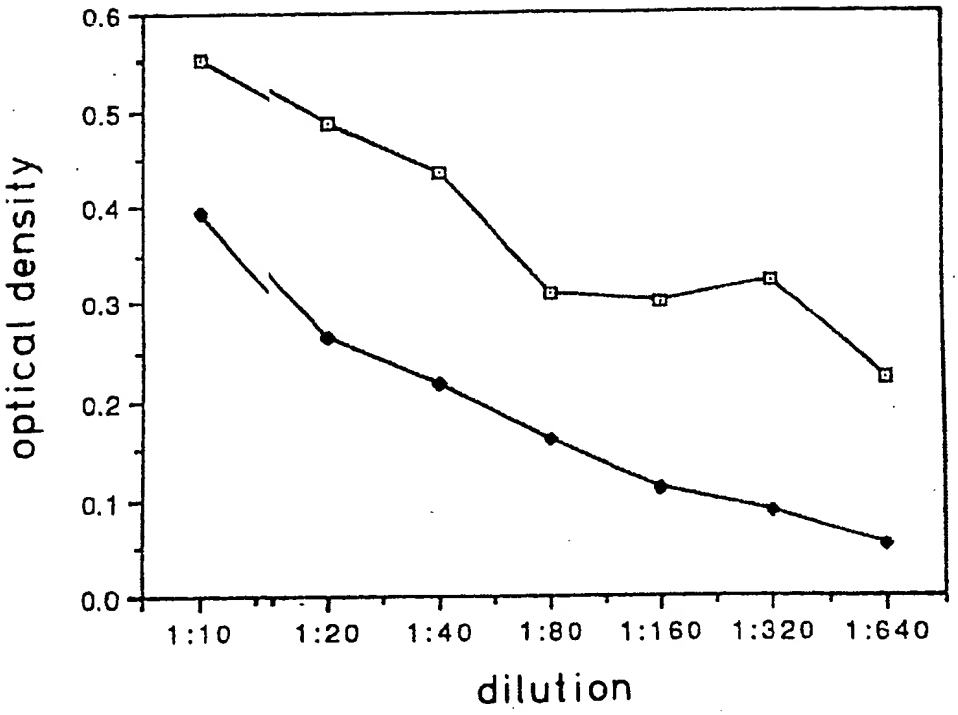

optical density
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1:10
1:20
1:40
1:80
1:160
1:320
1:640
dilution

Figure 8

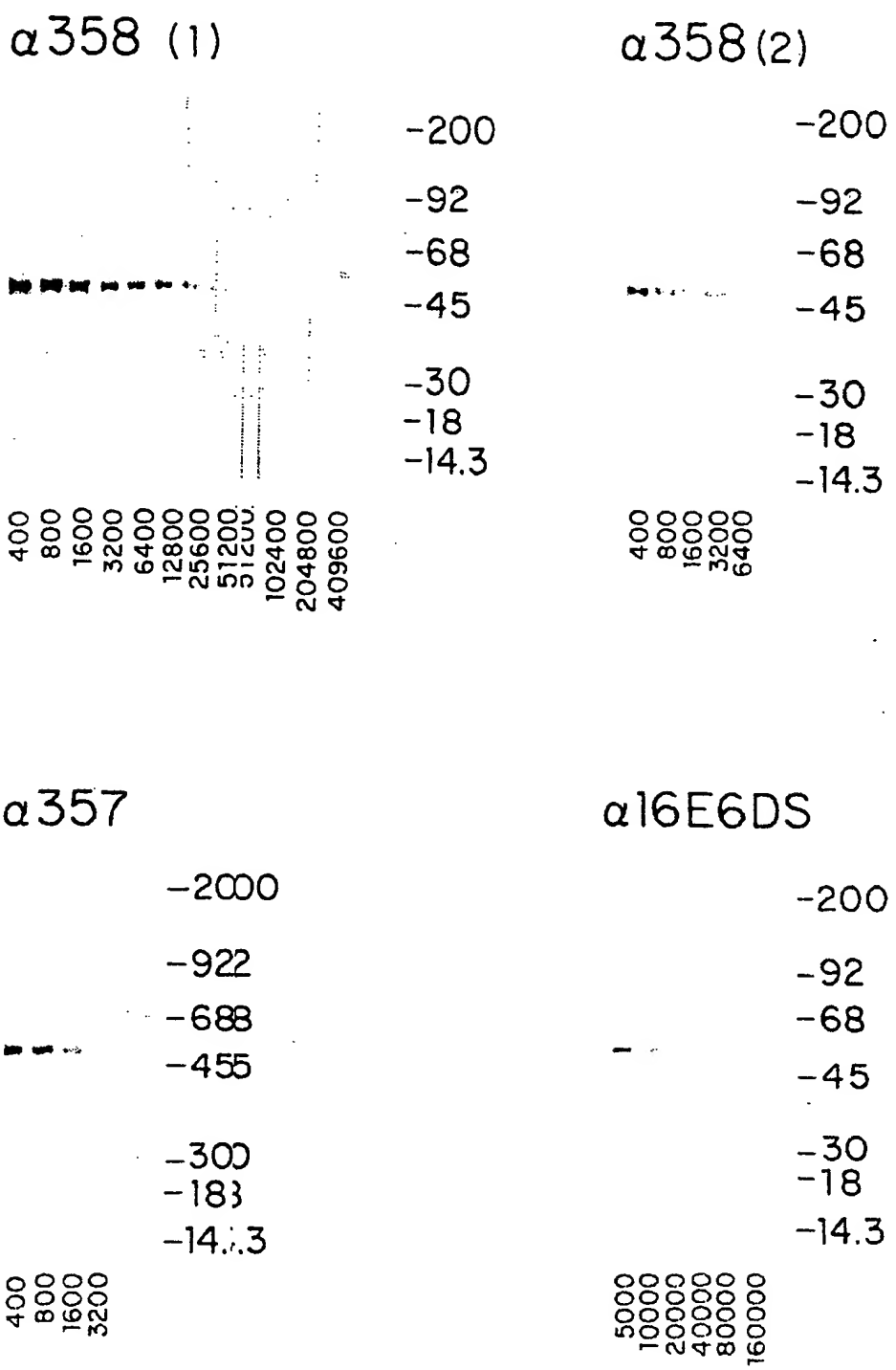

α358 (1)
-200
-92
-68
-45
-30
-18
-14.3
400
800
1600
3200
6400
12800
25600
51200
102400
204800
409600
α358 (2)
-200
-92
-68
-45
-30
-18
-14.3
400
800
1600
3200
6400
α357
-2000
-922
-688
-455
-300
-183
-14.3
400
800
1600
3200
α16E6DS
-200
-92
-68
-45
-30
-18
-14.3
5000
10000
20000
40000
80000
160000

Figure 9

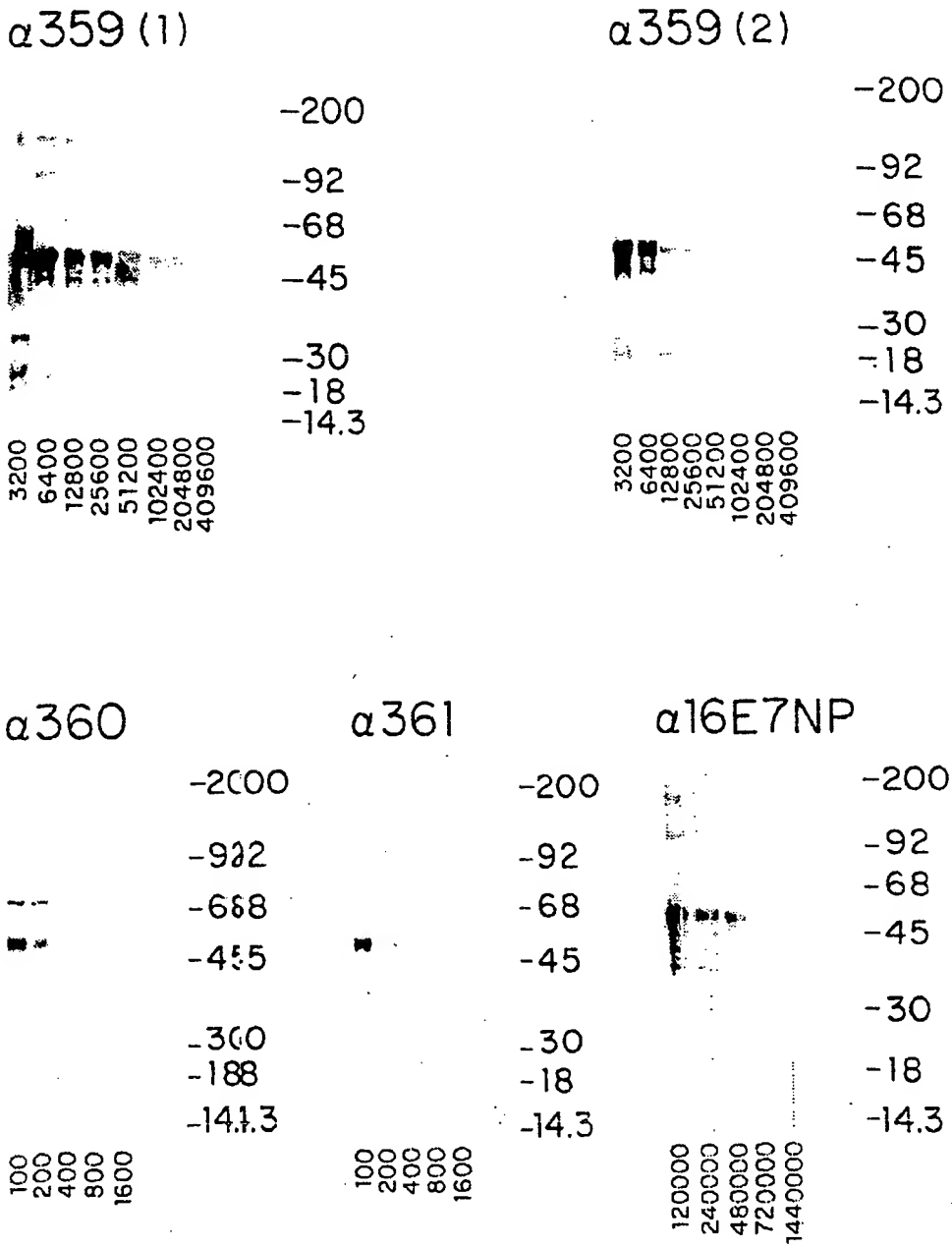

α359 (1)
-200
-92
-68
-45
-30
-18
-14.3
3200
6400
12800
25600
51200
102400
204800
409600
α359 (2)
-200
-92
-68
-45
-30
-18
-14.3
3200
6400
12800
25600
51200
102400
204800
409600
α360
-200
-92
-68
-45
-30
-18
-14.3
100
200
400
800
1600
α361
-200
-92
-68
-45
-30
-18
-14.3
100
200
400
800
1600
α16E7NP
-200
-92
-68
-45
-30
-18
-14.3
120000
240000
480000
720000
1440000

Figure 10

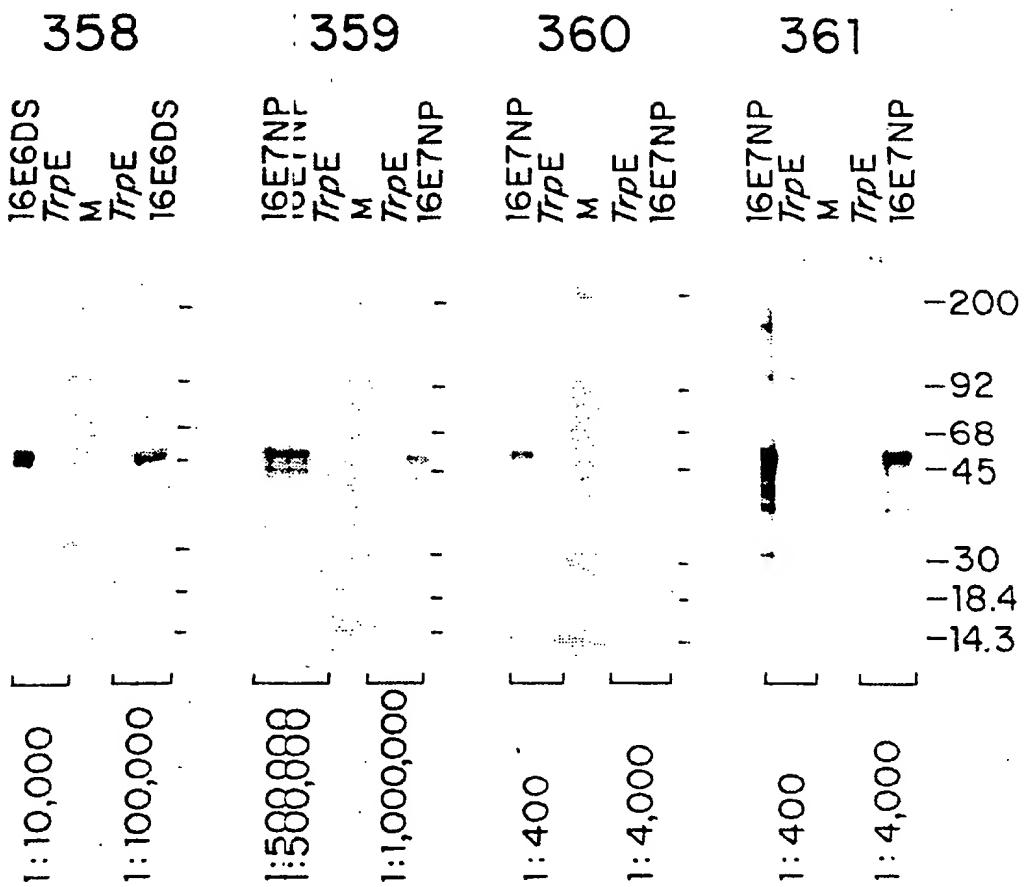



Figure 11

SUBSTITUTE SHEET

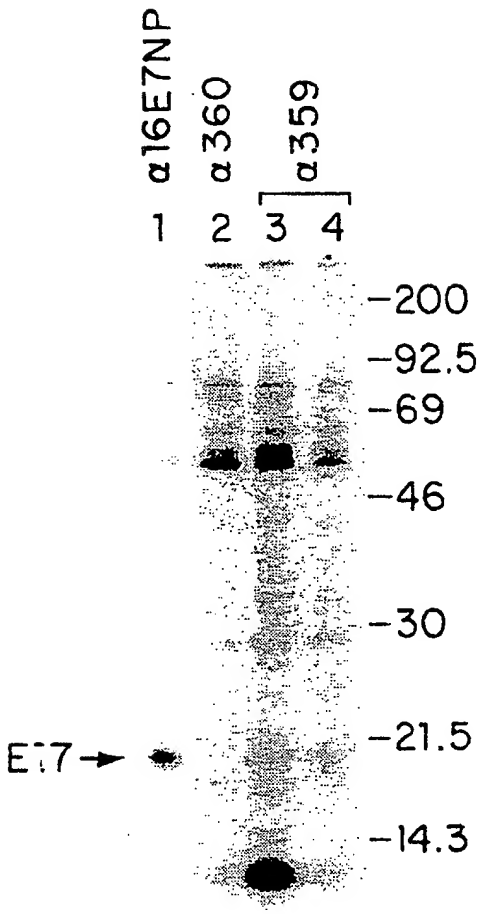



Figure 12

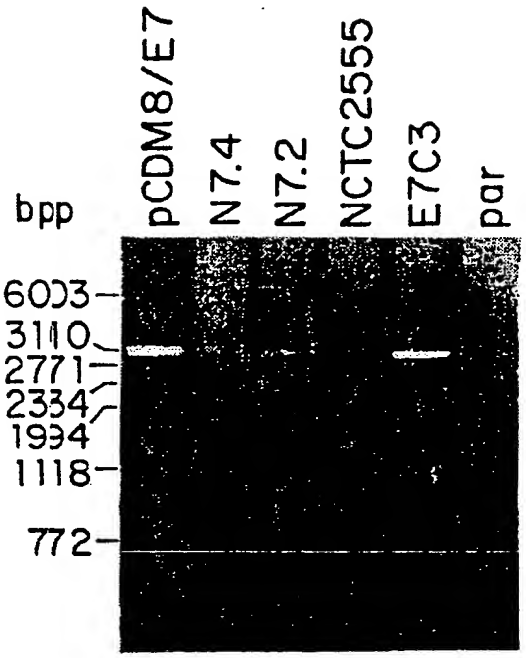

bpp
pCDM8/E7
N7.4
N7.2
NCTC2555
E7C3
par
6003
3110
2771
2334
1934
1118
772

Figure 13

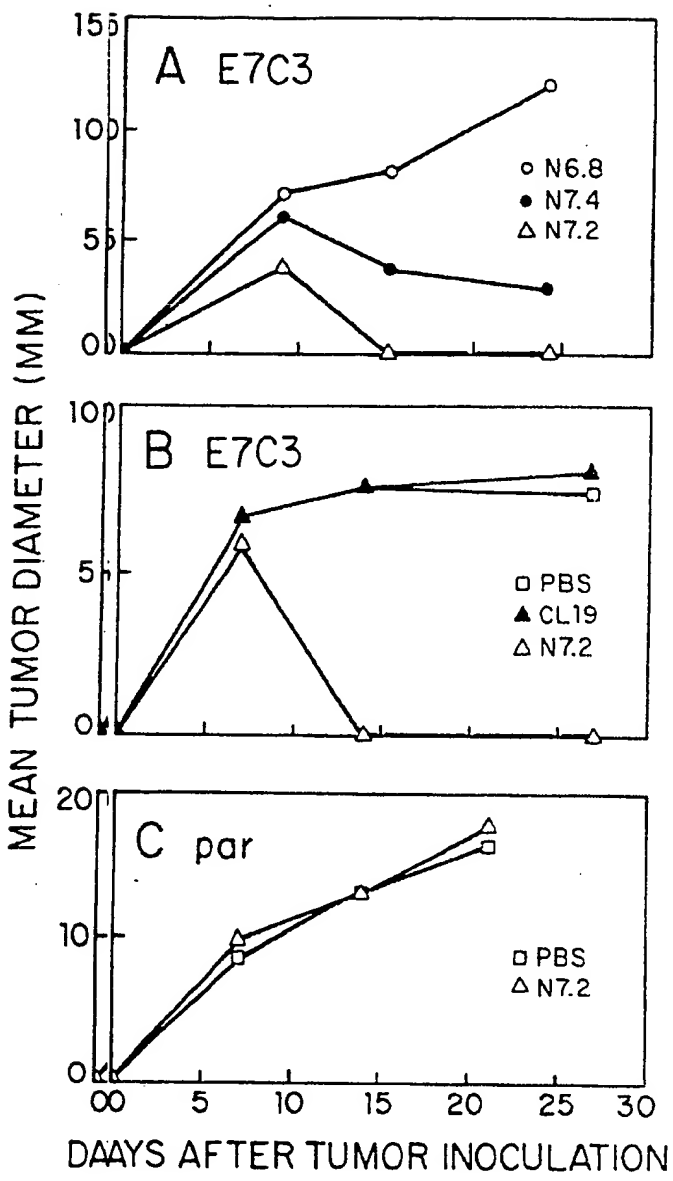

A E7C3
N6.8
N7.4
N7.2
B E7C3
PBS
CL19
N7.2
C par
PBS
N7.2
MEAN TUMOR DIAMETER (MM)
DAAYS AFTER TUMOR INOCULATION

Figure 14

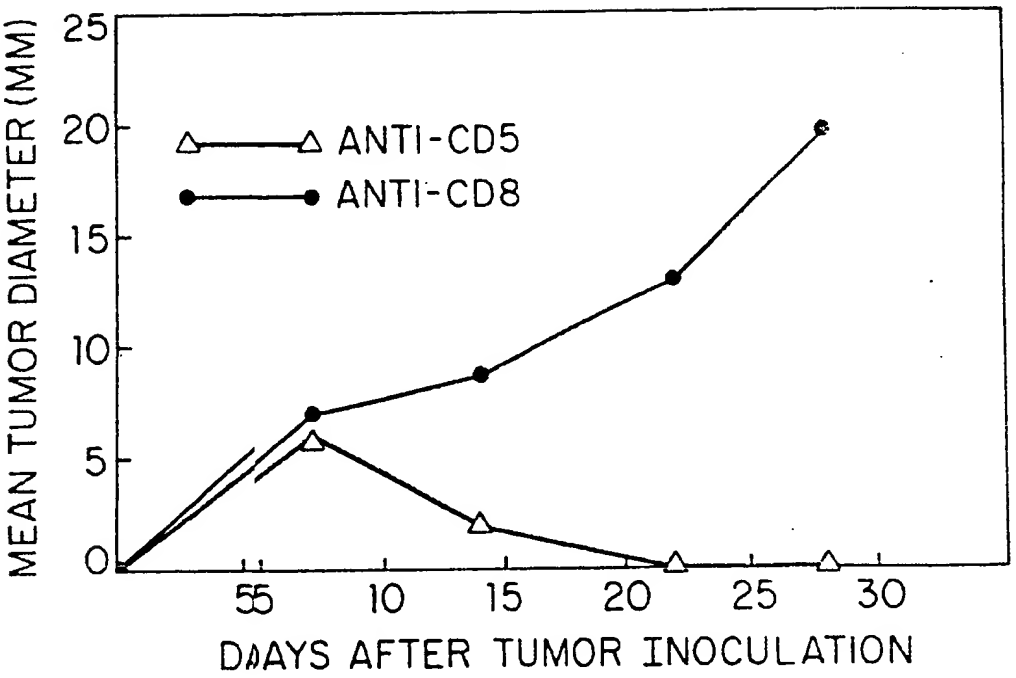

MEAN TUMOR DIAMETER (MM)
25
20
15
10
5
0
ANTI-CD5
ANTI-CD8
55
10
15
20
25
30
DAYS AFTER TUMOR INOCULATION

Figure 15

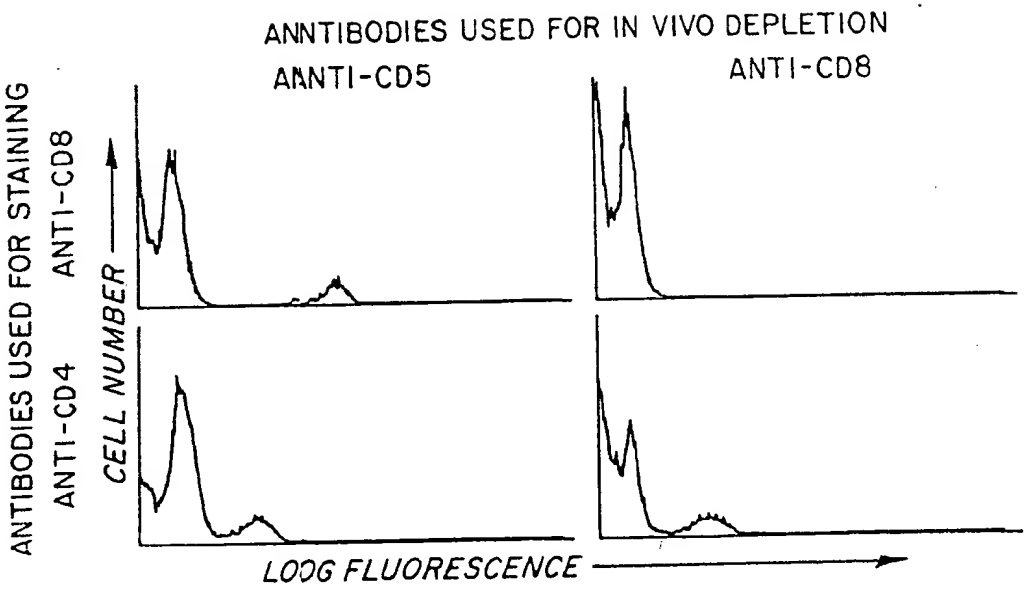

ANNTIBODIES USED FOR IN VIVO DEPLETION
ANNTI-CD5
ANTI-CD8
ANTIBODIES USED FOR STAINING
ANTI-CD8
ANTI-CD4
CELL NUMBER
LOOG FLUORESCENCE

Figure 16

# INNTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/07081

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classsification (IPC) or to both National Classification and IPC

IPC(5):C12N 7/00, 77/04; 15/00, 15/37. 15/86
U.S.CL.;435/69.1, 2235, 236, 320.1

## II. FIELDS SEARCHED

Minimum Documentation Searched [7]

| Classification System | Classification Symbols |
|---|---|
| U.S. | 435/69.1, 235, 236, 239, 240.2, 240.23, 320.1; 935/32.1, 57, 71 |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

APS; Dialog Databasses; Biosis, Embase, Meline; Genbank

## III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

| Category * | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No. [13] |
|---|---|---|
| Y | Virology. Vol. 145. issued 1985. K. Seedorf et al. "Human Papillomavirus Type 16 DNA Sequence". pages 181-185. See entire document. | 1-23.25-54 |
| Y | J. Virol.. vol. 61. no. 11. issued November 1987. M.A. Bedell et al.. "The E6-E7 Region of Human Papillomavirus Type 18 is Sufficient for Transformation of NIH 3T3 and Rat-1 cells". pages 3635-3640. See page 3639. | 1-25. 38-48 |
| Y | Vaccine. vol. 8. no. 3. issued June 1990. G. Meeneguzzi et al.. "Vaccinia Recombinants Expressing Early Bovine Papilloma Virus (BPVI) Proteins: Retardation of BPVI Tumour Development". pages 199-2204. See entire article. | 1-60 |

* Special categories of cited documments: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 08 January 1992 | 29 JAN 1992 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| ISA/US | Dian Cook for ebw |

Form PCT/ISA/210 (second sheet) (Rev.11-87)